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Regulación de la activación de macrófagos alveolares por la proteína del surfactante pulmonar SP-A

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PRESENTADA POR

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I. ABBREVIATIONS

aMϕ´s: Alveolar macrophages

BAL: Bronchoalveolar lavage

C1q: First subcomponent of the C1 complex of complement activation

CXCL10: C-X-C motif chemokine 10

ETV7: Transcription factor ETV7

i.p.: Intra-peritoneal

IFN-γR1: Interferon gamma receptor 1 or CD119

IL-: Interleukin

IL-4c: IL-4 complex

IL-4Rα: Interleukin 4 receptor alpha

M(IL-4) or M2: Macrophage activated via the IL-4Rα

M(LPS/ IFN-γ) or M1: Macrophage activated by LPS and/or IFN-γ

MBP: Mannose binding protein

MFI: Mean fluorescence intensity

Myo18A: Unconventional cell surface myosin18A

Mϕ: Macrophage

Mϕ(GM-CSF): human GM-CSF-derived macrophages

Mϕ(M-CSF): human M-CSF-derived macrophages

pMϕs: Peritoneal macrophages

RARRES3: Retinoic acid receptor responder 3

RELMα: Resistin like protein alpha

sIFN-γR1: soluble fraction of interferon gamma receptor 1

SP-: Surfactant protein

TGF-β: Transforming growth factor beta

TNF-α: Tumor necrosis factor alpha

WT: Wild type

Ym1: Chitinase-like protein 3

II. RESUMEN

Introducción

Para facilitar el intercambio gaseoso, el pulmón tiene la mayor área del cuerpo en contacto con el medio externo. Se estima que hay alrededor de 300 millones de alvéolos en pulmones humanos adultos, lo que supone un área de contacto con el exterior 60 veces mayor que la superficie de la piel. Esta extensa superficie de contacto con el medio externo facilita la oxigenación, pero aumenta el riesgo de infección e inflamación por patógenos y endotoxinas presentes en el aire que respiramos. De hecho, el epitelio respiratorio es, con frecuencia, la puerta de entrada de microorganismos y alérgenos causantes de numerosas enfermedades.

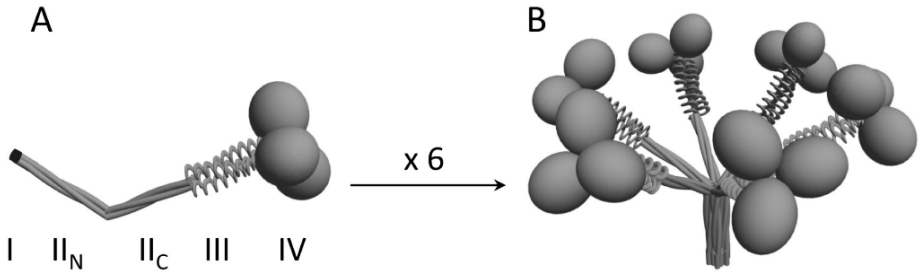
El sistema inmune innato en los alvéolos se caracteriza por una respuesta inmune humoral (lactoferrina, lisozima, colectinas pulmonares (SP-A y SP-D), β-defensinas y otros péptidos antimicrobianos) y celular, constituida principalmente por los macrófagos alveolares que coordinan la defensa del huésped [1]. Los macrófagos alveolares promueven la tolerancia a antígenos inocuos presentes en el aire inhalado, reduciendo la respuesta inflamatoria que podría comprometer la integridad de pulmón [1, 2]. Sin embargo, durante una infección, los macrófagos reconocen señales de alarma a través de receptores capaces de inducir programas de activación especializados. La activación clásica de los macrófagos (M1) es inducida por IFN-γ y patrones moleculares asociados a patógenos que son ligandos de TLR [1, 2]. Sin embargo, los macrófagos también adquieren un programa de activación alternativa (M2) en respuesta a IL-4 e IL-13, importante para la respuesta inmune ante infecciones parasitarias y para la reparación del tejido [3-6].

Los macrófagos tienen una extraordinaria plasticidad para adaptarse a cambios en su entorno. Los macrófagos activados por la vía clásica (M1) tienen potentes propiedades microbicidas y promueven una fuerte respuesta proinflamatoria, mientras que los macrófagos activados alternativamente (M2) tienen alta actividad fagocítica, promueven la remodelación del tejido y reducen la secreción de citoquinas proinflamatorias [4]. La flexibilidad de los macrófagos alveolares de cambiar de un estado de activación a otro podría ser importante para mantener los alvéolos estériles y poco inflamados. La alteración o desregulación de este balance M1↔M2 podría estar asociada a enfermedades respiratorias ya que la persistencia de una respuesta pro-inflamatoria (M1) es perjudicial para el tejido [4, 7] y, al contrario, la permanencia de un fenotipo de activación alternativa (M2) favorece la infección por bacterias y virus,

el progreso de tumores y el desarrollo de determinadas enfermedades respiratorias crónicas como la fibrosis pulmonar [4, 8].

Los macrófagos alveolares se encuentran en un entorno único. Están cubiertos por un fluido acuoso enriquecido en un material lipoproteico denominado surfactante pulmonar, que es esencial para mantener los alveolos abiertos durante los ciclos de inspiración-espiración [9]. La carencia o alteración del surfactante pulmonar ya sea debido a inmadurez pulmonar por nacimiento prematuro, daño pulmonar agudo, o mutación en genes críticos para la producción y función del surfactante, causa fallo respiratorio [10]. El surfactante pulmonar no sólo protege al pulmón del colapso alveolar, sino que también participa en la defensa inmune innata [9], especialmente debido a las proteínas SP-A y SP-D del surfactante [11]. El potencial efecto de los componentes del surfactante, y en concreto de la SP-A, sobre la polarización de macrófagos alveolares hacia un fenotipo pro-inflamatorio M1 o un fenotipo anti-inflamatorio M2 no ha sido investigado en profundidad.

La proteína SP-A es una proteína oligomérica, estructuralmente similar a la proteína del complemento C1q. Pertenece a la familia de las colectinas por poseer dominios fibrilares de tipo colágeno que agrupan tres dominios globulares con actividad de lectina tipo-C dependiente de calcio.



Modelo tridimensional de la forma trimétrica (A) y oligomérica (B) de la SP-A. En (A) se muestran los cuatro dominios estructurales de la cadena polipeptídica de la SP-A humana: I) Segmento NH_2-terminal; II) dominio de colágeno con una irregularidad en la secuencia que divide dicho dominio en dos pates: NH_2-terminal (II_N) y COOH-terminal (II_C); III) región del cuello entre el dominio colágeno y el globular, que forma una estructura de *coiled coil*; y IV) dominio globular COOH-terminal con dominio de reconocimiento de carbohidratos. Figura modificada de [9].

Miembros de esta familia son la SP-D, la proteína de unión a manosa (MBP) o la adiponectina. La SP-A se une a lípidos del surfactante y a una gran variedad de

ligandos inmunes y no inmunes presentes en el fluido alveolar y en la superficie de células alveolares. Las principales funciones de la SP-A son: 1) regular la inflamación alveolar mediante su unión a distintos receptores en la superficie de células del epitelio alveolar, macrófagos y linfocitos [11] y favorecer la fagocitosis de células apoptóticas por macrófagos alveolares contribuyendo a la resolución de la inflamación [12]; y 2) controlar la infección. Para llevar a cabo esta segunda función, la SP-A aumenta la fagocitosis de patógenos por macrófagos alveolares y/o la expresión de receptores implicados en reconocimiento de patógenos, como el receptor de manosa [11, 13]. Además, la SP-A tiene una actividad microbicida directa en combinación con péptidos antimicrobianos presentes en el fluido alveolar [14] lo que explica que los lavados broncoalveolares libres de células de ratones SP-$A^{+/+}$ tengan un efecto bactericida mucho mayor que los lavados provenientes de ratones SP-$A^{-/-}$ [15].

Objetivo

El objetivo principal de esta tesis fue evaluar el efecto de la SP-A sobre la polarización de macrófagos alveolares hacia un fenotipo pro-inflamatorio M1 o un fenotipo anti-inflamatorio M2 durante la activación clásica y alternativa de macrófagos, respectivamente.

Esta tesis consta de tres capítulos de resultados con los siguientes objetivos específicos:

1. Evaluar el efecto de la SP-A en el fenotipo clásico de los macrófagos alveolares inducido por IFN-γ en presencia o ausencia de LPS.

2. Determinar el papel de la SP-A en la activación alternativa y proliferación de macrófagos alveolares mediada por IL- 4Rα e investigar si la SP-A podría ser un factor específico de tejido que regula la capacidad de los macrófagos de responder en mayor o menor media a IL-4.

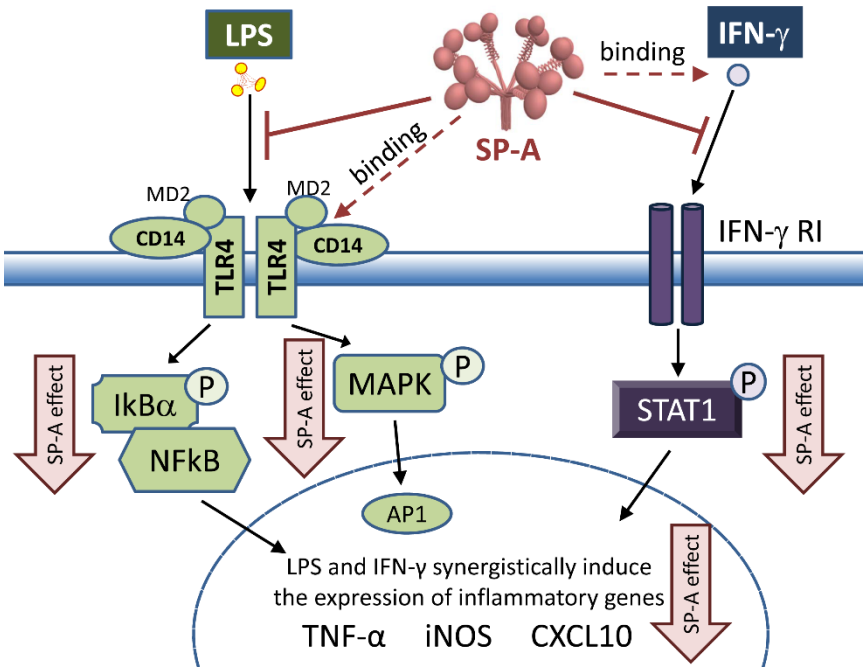
3. Estudiar el mecanismo por el cual la SP-A incrementa la proliferación y activación de macrófagos alveolares inducida por IL-4.

Resultados

Capítulo 1

Para determinar el efecto de la SP-A sobre macrófagos activados por la vía clásica, se aislaron macrófagos alveolares a partir de pulmones humanos y de rata, y se estimularon con IFN-γ y LPS simultáneamente o por separado, en presencia y ausencia de SP-A.

Se observó que la SP-A inhibió la producción de TNF-α, iNOS y CXCL10 por macrófagos alveolares de rata estimulados con IFN-γ y LPS. Cuando las células se estimularon con LPS e IFN-γ por separado, la SP-A inhibió tanto la fosforilación de ERK inducida por LPS como la fosforilación de STAT1 inducida por IFN-γ. La SP-A también disminuyó la secreción de TNF-α y CXCL10 por macrófagos alveolares humanos cultivados *ex vivo* y macrófagos derivados de monocitos humanos con M-CSF [Mϕ(M-CSF)] estimulados con LPS y/o IFN-γ. Además, la SP-A inhibió la transcripción de genes regulados por IFN-γ (*CXCL10*, *RARRES3* y *ETV7*), así como la fosforilación de STAT1 en Mϕ(M-CSF). Finalmente, demostramos que la SP-A se unió al IFN-γ humano (K_D =11 ± 0.5 nM) de manera dependiente de Ca^{2+} lo que impidió la interacción entre IFN-γ con su receptor IFN-γR1 en la superficie celular de macrófagos alveolares humanos.



Conclusiones del capítulo 1: Los resultados de este capítulo nos permiten concluir que la SP-A limita la activación clásica de macrófagos alveolares humanos y de rata inducida por IFN-γ y/o LPS. La acción de la SP-A en macrófagos activados por IFN-γ y LPS se basa en su capacidad para atenuar ambos agentes inflamatorios. Mientras que el mecanismo por el cual la SP-A limita la activación de macrófagos inducida por LPS se ha descrito anteriormente [16, 17], los resultados de este capítulo demuestran, por vez primera, que la SP-A se une al IFN-γ con alta afinidad, inhibiendo el reconocimiento de IFN-γ por su receptor en la superficie celular de macrófagos alveolares humanos. Por este mecanismo la SP-A podría aumentar el umbral de activación de macrófagos alveolares, evitando una respuesta inflamatoria ante agentes inocuos presentes en el aire inalado o facilitando la resolución de una respuesta inflamatoria.

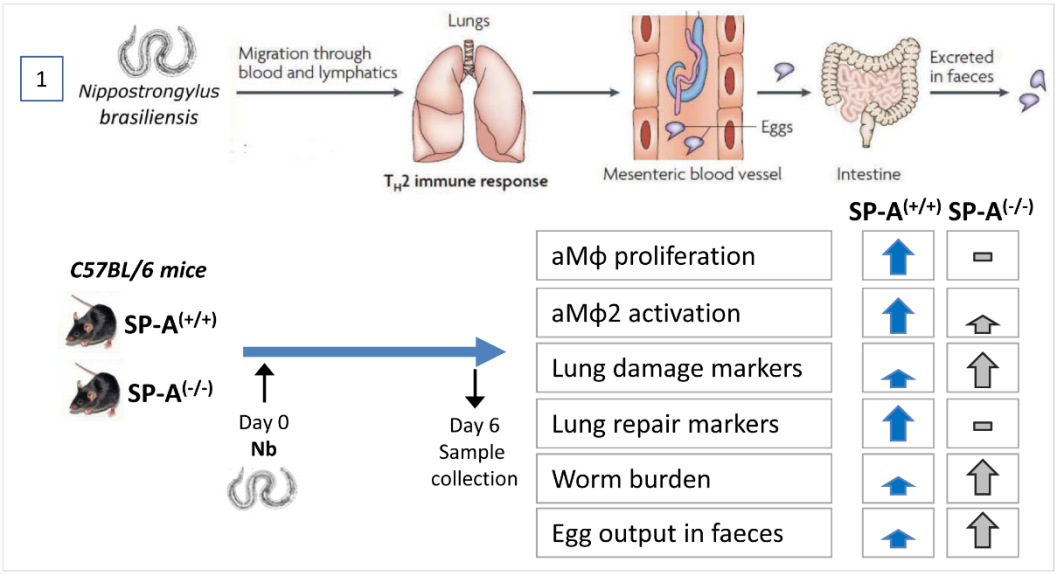
Capítulo 2

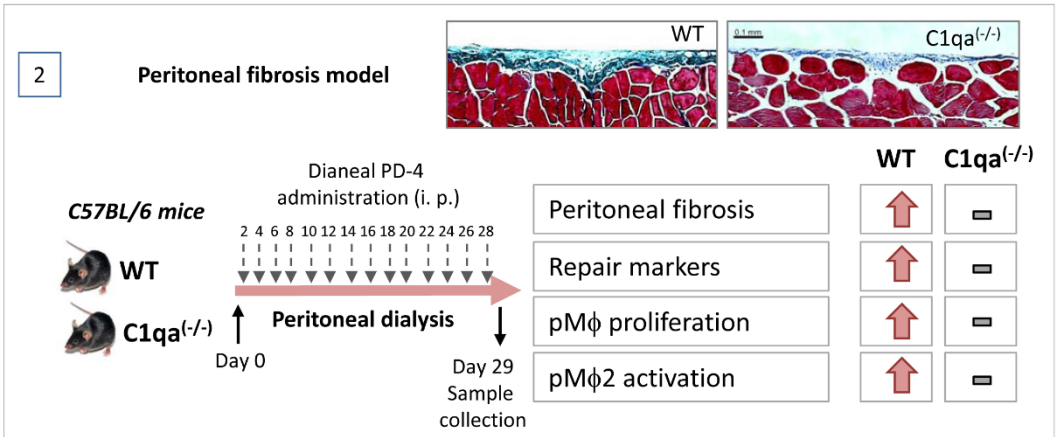
Para determinar el efecto de la SP-A en la activación alternativa de macrófagos inducida por IL-4, se aislaron macrófagos alveolares a partir de pulmones humanos, de ratón y rata, y se estimularon con IL-4 en presencia y ausencia de SP-A. Alternativamente, se comparó la respuesta de macrófagos alveolares en ratones *wild type* y ratones deficientes en SP-A tratados con IL-4c exógena. Por último, la relevancia fisiológica del efecto de la SP-A en la activación alternativa de macrófagos alveolares se evaluó en un modelo de infección por el parasito *Nippostrongylus brasiliensis*.

Se observó que la SP-A aumentó la activación y proliferación de macrófagos alveolares inducida por IL-4 *in vitro* e *in vivo*. Del mismo modo, la SP-A aumentó la activación y proliferación de los macrófagos alveolares en respuesta a la infección por el parasito *N. brasiliensis*, lo que contribuyó fundamentalmente a la eliminación del parásito y a la resolución del daño pulmonar causado por la migración del nematodo a través del pulmón. Además, en este capítulo identificamos a la miosina no convencional Myo18A (o SP-R210) como el receptor de la SP-A implicado en el aumento de la activación y proliferación de los macrófagos alveolares dependiente de IL-4 ya que el silenciamiento o bloqueo de dicho receptor (*in vitro* e *in vivo*) anula los efectos de la SP-A.

Para determinar si la SP-A es un factor específico de tejido, que incrementa la capacidad de los macrófagos de responder a IL-4, se realizaron experimentos con macrófagos aislados de la cavidad peritoneal y con una proteína estructuralmente análoga a SP-A, la C1q. SP-A incrementó la activación y proliferación dependiente de IL-4 de macrófagos alveolares, pero no peritoneales, mientras que la C1q actuó de forma similar a la SP-A en macrófagos peritoneales, pero no alveolares. Experimentos *in vivo* con ratones C1qa$^{-/-}$ demostraron que C1q refuerza la activación y la proliferación de macrófagos mediada por IL-4 en la cavidad peritoneal también a través del receptor Myo18A. Este efecto de la C1q promovió el desarrollo de fibrosis peritoneal en un modelo de diálisis peritoneal en ratón, cuyo líquido de diálisis contiene lactato.

Nuestros resultados indican que SP-A y C1q son amplificadores locales de la respuesta inmune tipo 2 y sugieren que otros colágenos de defensa (MBP o adiponectina) podrían tener la misma acción en otros tejidos. El hecho de que el mismo receptor Myo18A esté mediando funciones tejido-específicas para SP-A y C1q,



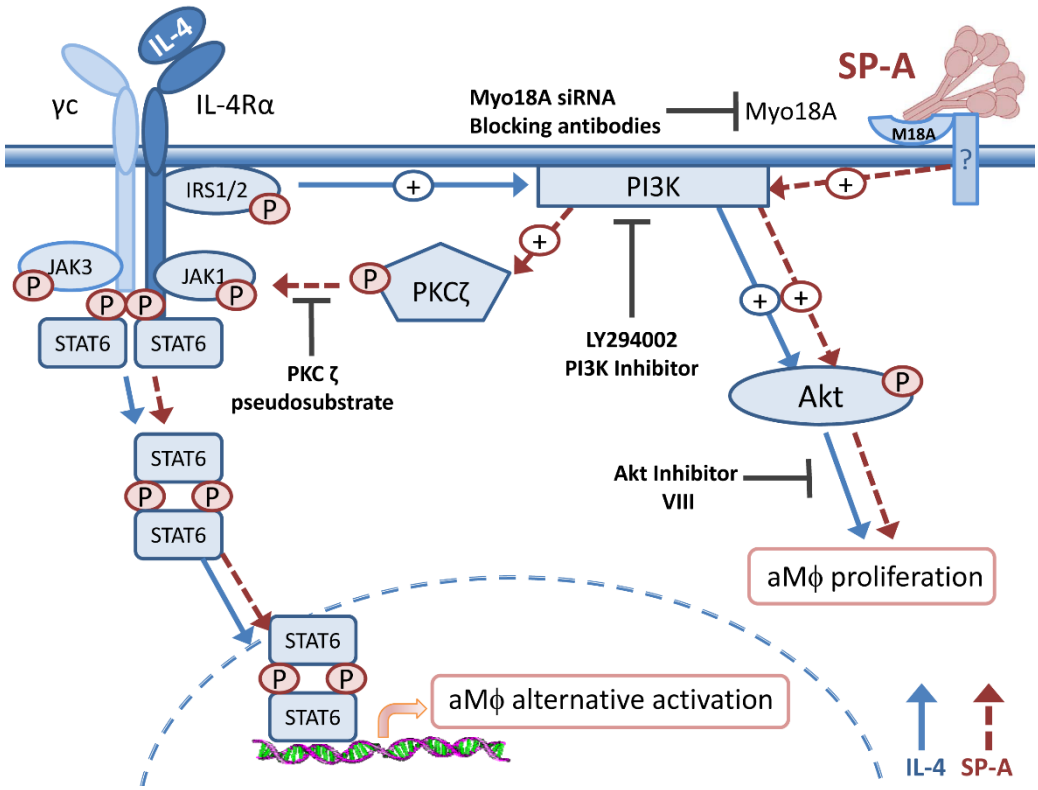


sugiere la existencia de co-receptores que actúen en concierto con Myo18A y que determinan la especificidad de tejido.

Conclusiones del capítulo 2: la SP-A en el pulmón y la C1q en la cavidad peritoneal son factores específicos de tejido que actúan a través del receptor Myo18A para amplificar la activación y la proliferación de macrófagos residentes mediada por IL-4Rα. La utilización de un modelo de infección pulmonar por nematodos indica que la acción amplificadora de la SP-A tiene consecuencias en la reparación del tejido dañado y en el control de la infección por el parásito. Por otra parte, la utilización de un modelo de diálisis peritoneal indica que la acción amplificadora de la C1q sobre la proliferación y activación alternativa de macrófagos peritoneales tiene consecuencias en la progresión de la fibrosis peritoneal inducida por diálisis peritoneal.

Capítulo 3

Para determinar los mecanismos moleculares por los que la SP-A amplifica la activación y la proliferación de macrófagos alveolares mediada por IL- 4, las células se trataron con IL-4 y SP-A en presencia de inhibidores farmacológicos de las rutas de señalización disparadas por IL-4 o involucradas en la potencial señalización de SP-A.



Conclusiones del capítulo 3: Los resultados permiten concluir que SP-A, a través de la unión al receptor Myo18A, activa PI3K y desencadena la fosforilación de Akt y PKCζ. Por un lado, SP-A amplifica la vía de señalización de PI3K-Akt inducida por IL-4, que está implicada en la proliferación de los macrófagos. Por otro lado, el eje SP-A/Myo18A/PI3K/PKCζ está implicado en incrementar la fosforilación de STAT6 necesaria para la activación alternativa de macrófagos.

La acción de SP-A es dependiente de su unión al receptor Myo18A por el dominio de colágeno, por lo que la expresión de Myo18A en la superficie celular podría ser una diana terapéutica para el tratamiento intratraqueal de patologías causadas por un descontrol en la activación de macrófagos alveolares hacia la vía alternativa.

Conclusión

Los resultados de esta tesis permiten concluir que la proteína del fluido alveolar SP-A modula la activación de macrófagos alveolares, limitando su activación clásica, inducida por IFN-γ, y promoviendo su activación alternativa y proliferación en presencia de IL-4.

III. SUMMARY

Introduction

To facilitate gas exchange, the lung has the largest area of the body in contact with the external environment. It is estimated that there are about 300 million alveoli in the adult human lungs, representing an area of contact with the environment 60 times larger than the surface of the skin. This massive area of contact with the environment facilitates oxygenation but increases the risk of infection and inflammation by pathogens and endotoxins present in the air we breathe. In fact, the lung epithelium is often the gateway for microorganisms and allergens that cause many respiratory diseases.

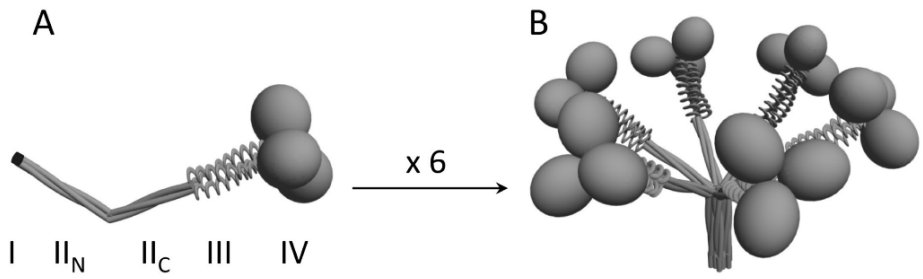
The innate immune system in the alveoli is made up of a humoral arm (lactoferrin, lysozyme, pulmonary collectins (SP-A and SP-D), β-defensins, and other antimicrobial peptides) and a cellular arm dominated mainly by alveolar macrophages, which coordinate host defence [1]. Alveolar macrophages promote tolerance to innocuous antigens present in the inhaled air, reducing the inflammatory response, which could compromise the integrity of the lung [1, 2]. However, during an infection, macrophages recognise alarm signals through receptors capable of inducing specialised activation programs. Classical macrophage activation (M1) is induced by IFN-γ and pathogen-associated molecular patterns that are TLR ligands [1, 2]. However, macrophages also acquire an alternative activation program (M2) in response to IL-4 and IL-13, which is important for the immune response to parasite infections and tissue repair [3-6].

Macrophages have an extraordinary plasticity to adapt to changes in their environment. Classically activated macrophages (M1) have potent microbicidal properties and promote a strong pro-inflammatory response, whereas alternatively activated macrophages (M2) have high phagocytic capacity, tissue remodelling activity, and reduced pro-inflammatory cytokine secretion [4]. The plasticity of alveolar macrophages in changing from one activation state to another could be important to keep the alveoli sterile and not inflamed. Alteration or dysregulation of this balance M1↔M2 might be associated with respiratory diseases since the persistence of a pro-inflammatory response (M1) is harmful to the tissue [4, 7] and, conversely, the permanence of an alternatively activated phenotype (M2) favours bacterial and viral

infections, promotes tumour progress and the development of certain chronic respiratory diseases such as pulmonary fibrosis [4, 8].

Alveolar macrophages are located in a unique environment. They are covered by an aqueous fluid enriched in a lipid-protein material called pulmonary surfactant, which is essential to keep the alveoli open during cycles of inhalation-exhalation [9]. The lack or alteration of pulmonary surfactant, whether caused by premature birth, acute lung injury, or mutations in genes critical to surfactant production or function, causes respiratory failure [10]. Pulmonary surfactant not only protects the lung from alveolar collapse, but also participates in the innate immune defence [9], especially due to the activity of surfactant proteins SP-A and SP-D [11]. The potential effect of surfactant components, in particular of SP-A, on alveolar macrophage polarisation into either pro-inflammatory (M1) or anti-inflammatory (M2) phenotype has not been investigated in depth.

SP-A is a large oligomeric protein, structurally similar to the first component of the complement system (C1q). SP-A belongs to the collectin family, which show collagen-type fibrillar domains and globular domains with calcium-dependent C-type lectin activity.



Three-dimensional model of the trimeric (A) and oligomeric (B) form of SP-A. In (A) the four structural domains of the polypeptide chain of human SP-A are shown: I) N-terminal segment; II) collagen-like domain characterized by a sequence irregularity (kink), which divides the collagen-like domain into two parts: N-terminal (II_N) and C-terminal (II_C) portions; III) neck region between the collagen and the globular domain, forming a coiled coil structure; and IV) C-terminal globular domain containing a carbohydrate recognition domain. Modified from [9].

Members of this family include SP-D, mannose binding protein (MBP), and adiponectin. SP-A binds to surfactant lipids and to a great variety of immune and non-immune ligands, present in the alveolar fluid and alveolar cells. The main functions of SP-A are: 1) to regulate alveolar inflammation by binding to different receptors on the

surface of alveolar cells [11] and by promoting phagocytosis of apoptotic cells by alveolar macrophages contributing to the resolution of inflammation [12]; and 2) to control alveolar infections. To fulfil the latter function, SP-A enhances phagocytosis of pathogens by alveolar macrophages and/or up-regulates the expression of the mannose receptor involved in pathogen recognition [11, 13]. In addition, SP-A has a cooperative microbicidal action with antimicrobial peptides present in the alveolar fluid [14], which explains why cell-free bronchoalveolar lavages from SP-$A^{+/+}$ mice have a much greater bactericidal effect than that from $SPA^{-/-}$ mice [15].

Objective

The main objective of this thesis was to evaluate the effect of SP-A on alveolar macrophage polarisation towards a pro-inflammatory M1 or anti-inflammatory M2 phenotype during classical and alternative macrophage activation, respectively.

This thesis consists of three experimental chapters with the following specific objectives:

1. Evaluate the effect of SP-A on classical activation of alveolar macrophages induced by IFN-γ in the presence or absence of LPS.

2. Determine the role of SP-A during IL-4Rα-mediated alternative activation and proliferation of alveolar macrophages and investigate whether SP-A might be a tissue-specific factor that determines the ability of macrophages to respond to IL-4.

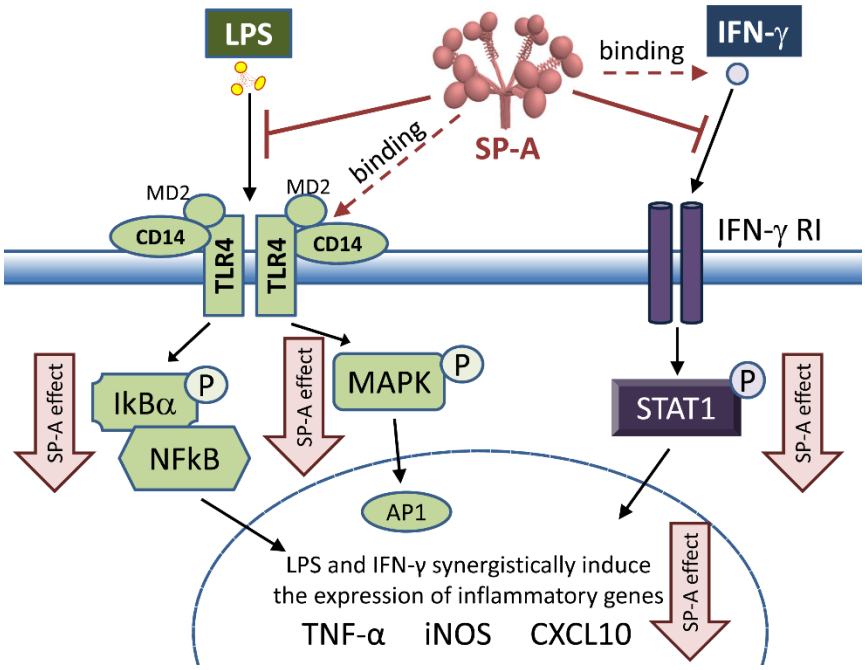
3. Study the mechanism by which SP-A enhances proliferation and activation of alveolar macrophages induced by IL-4.

Results

Chapter 1

To determine the effect of SP-A on macrophages activated by classical activation stimuli, alveolar macrophages were isolated from human and rat lungs, and cells were stimulated with IFN-γ and LPS simultaneously or separately, in the presence and absence of SP-A.

We observed that SP-A inhibited the production of TNF-α, iNOS, and CXCL10 by rat alveolar macrophages stimulated with IFN-γ and LPS. When cells were stimulated with LPS and IFN-γ separately, SP-A inhibited both LPS-induced ERK phosphorylation and STAT1 phosphorylation induced by IFN-γ. SP-A also decreased TNF-α and CXCL10 secretion by ex vivo cultured human alveolar macrophages and M-CSF-derived macrophages stimulated with LPS and/or IFN-γ. Hence, SP-A inhibited up-regulation of IFN-γ-inducible genes (CXCL10, RARRES3, and ETV7) as well as STAT1 phosphorylation in Mϕ (M-CSF). Finally, we demonstrated that SP-A bound to human IFN-γ (K_D = 11 ± 0.5 nM) in a Ca^{2+}-dependent manner and prevented IFN-γ interaction with IFN-γR1 receptor on the cell surface of human alveolar macrophages.



Conclusions of chapter 1: The results of this chapter allowed us to conclude that SP-A limits classical activation of rat and human alveolar macrophages induced by IFN-γ and LPS together or separately. The action of SP-A on [IFN-γ + LPS]-activated macrophages was based on its capability to attenuate both inflammatory agents. Whereas the mechanism by which SP-A limits LPS-induced activation of macrophages was previously described [16, 17], this chapter demonstrates for the first time that SP-A binds to IFN-γ with high affinity, inhibiting IFN-γ recognition by its receptor on the cell surface of alveolar macrophages. These data unravel a previously unknown mechanism by which SP-A/IFN-γ interaction plays a significant role in tipping the balance of inflammation to protect the alveolar epithelium and maintains a tolerant lung environment in the steady state.

Chapter 2

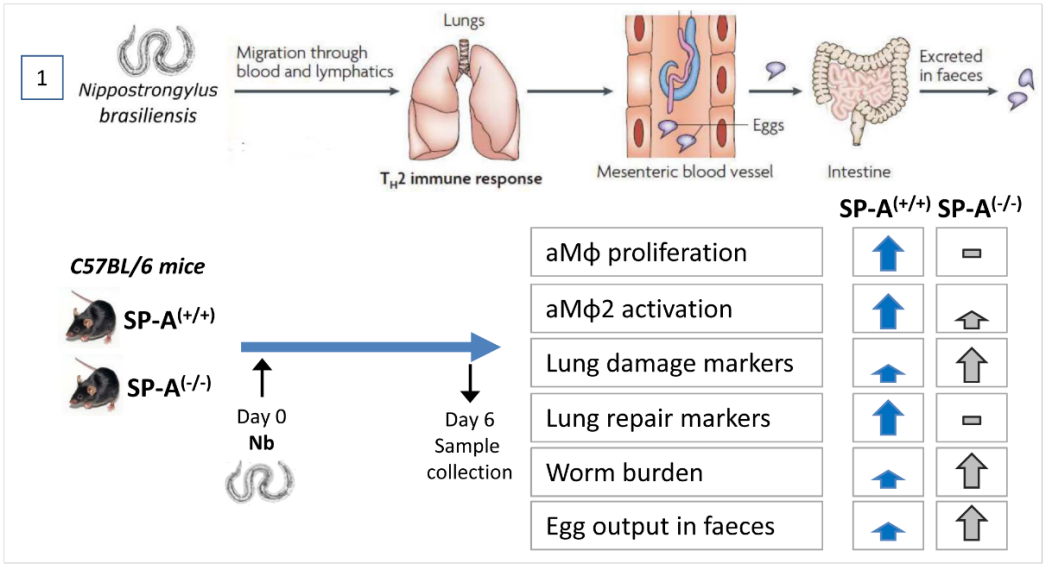
To determine the effect of SP-A on the alternative activation of alveolar macrophages induced by IL-4, alveolar macrophages were isolated from human, mouse, and rat lungs, and these cells were stimulated with IL-4 in the presence and absence of SP-A. Alternatively, we compared the response of alveolar macrophages from wild type vs. SP-A-deficient mice to exogenous IL-4 treatment *in vivo*. Finally, the physiological relevance of the effect of SP-A on the alternative activation of alveolar macrophages was assessed in a model of parasitic infection with *N. brasiliensis.*

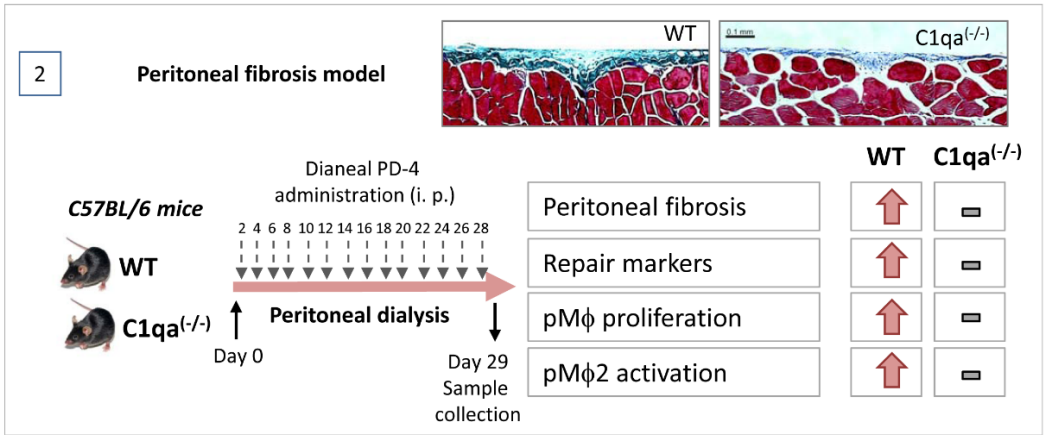
We found that SP-A enhanced IL-4-dependent activation and proliferation of alveolar macrophages *in vitro* and *in vivo*. Similarly, SP-A enhanced the activation and proliferation of alveolar macrophages in response to parasitic infection with *Nippostrongylus brasiliensis*. Critically, this contributed to the control of parasite infection and the resolution of lung injury caused by the lung-migrating nematode. We identified the unconventional myosin Myo18A (*aka* SP-R210) as the SP-A receptor involved in the enhancement of IL-4-dependent activation and proliferation of alveolar macrophages since silencing or blocking this receptor (*in vitro* and *in vivo*) abrogated SP-A's effects.

To determine whether SP-A might be a tissue-specific factor that could regulate the ability of macrophages to respond to IL-4, we performed experiments with macrophages isolated from the peritoneal cavity and with C1q, a protein structurally homologous to SP-A in its supra-trimeric assembly and collagen tail. We found that SP-

A significantly boosted IL-4-dependent activation and proliferation of alveolar but not peritoneal macrophages, whereas C1q had an effect similar to SP-A on peritoneal, but not alveolar macrophages. *In vivo* experiments with C1qa$^{-/-}$ mice demonstrated that C1q enhances IL-4-mediated activation and proliferation of macrophages in the peritoneal cavity, acting also through Myo18A receptor. Enhancement of peritoneal Mϕ alternative activation by C1q plays a relevant role in peritoneal fibrosis induced by a lactate dialysate used in the clinic. In this model, lactic acid seems to be a promoter of macrophage proliferation and Mϕ2 activation, and C1q amplified peritoneal macrophage response to lactate, enhancing the development of peritoneal fibrosis.

Our results indicate that SP-A and C1q are local amplifiers of the type 2 immune response and suggest that other defence collagens (MBP or adiponectin) might have the same action in other tissues. The fact that the same Myo18A receptor mediates tissue-specific functions of SP-A and C1q suggests the existence of co-receptors that act in concert with Myo18A to determine tissue specificity. Such molecules will be attractive candidates for tissue-targeted therapy.

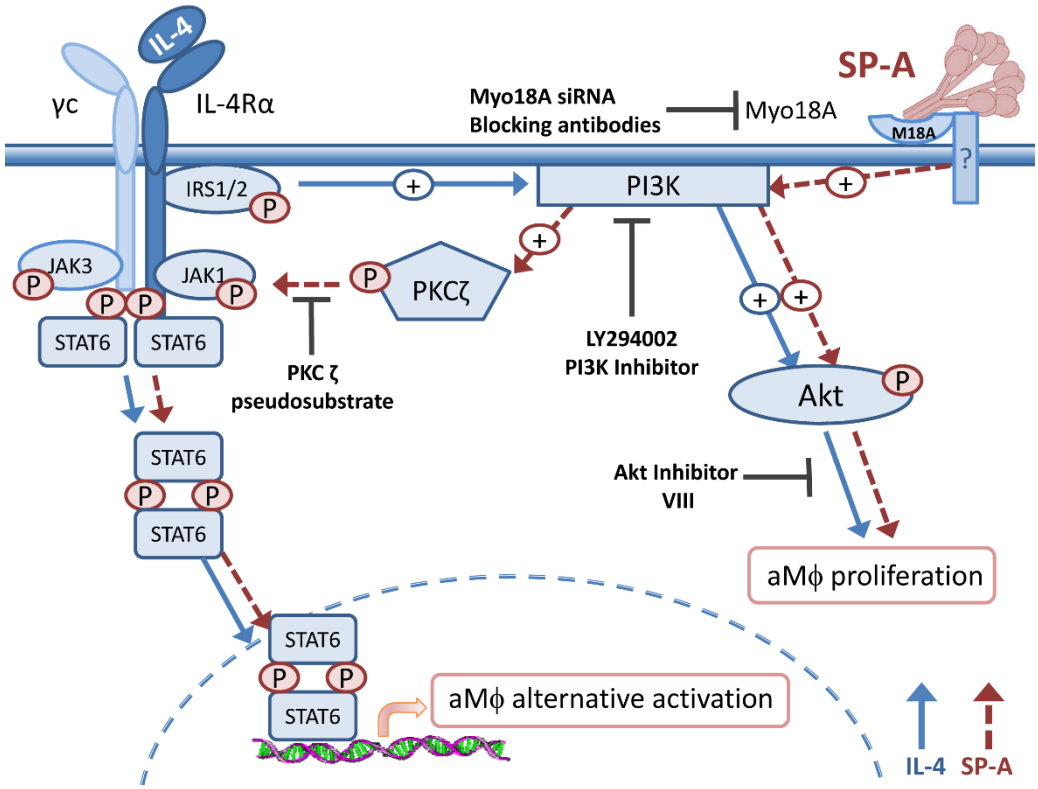




Conclusions of chapter 2: SP-A in the lung and C1q in the peritoneal cavity are tissue-specific factors that act through Myo18A receptor to amplify the activation and proliferation of resident macrophages mediated by IL-4Rα. Our experiments using a model of pulmonary nematode infection indicate that the amplifying action of SP-A has consequences for the control of parasite infection and the resolution of lung injury caused by the lung-migrating nematode. Moreover, the use of a model of peritoneal dialysis indicates that the amplifying action of C1q on proliferation and alternative activation of peritoneal macrophages has consequences on the progression of peritoneal fibrosis induced by peritoneal dialysis.

Chapter 3

To determine the molecular mechanisms by which SP-A amplifies the IL-4-mediated activation and proliferation of alveolar macrophages, these cells were treated with IL-4 and SP-A in the presence of pharmacological inhibitors of signalling triggered by IL-4 or SP-A.



Conclusions of chapter 3: Our results demonstrate that SP-A, through binding to Myo18A receptor, activates PI3K and induces subsequent phosphorylation of Akt and PKCζ downstream effectors. On the one hand, SP-A sustained the PI3K-Akt signalling pathway triggered by IL-4, which is involved in macrophage proliferation. On the other hand, SP-A/Myo18A/PI3K/PKCζ axis was involved in enhancing STAT6 phosphorylation, necessary for the alternative activation of macrophages.

The action of SP-A is dependent on its binding to Myo18A receptor through SP-A collagen domain. Therefore, the expression of Myo18A receptor on the surface of alveolar cells could be a target of potential therapeutic value for intra-tracheal treatment of diseases caused by an imbalanced alternative activation of alveolar macrophages.

Conclusion

The results of this thesis allow us to conclude that the alveolar fluid protein SP-A modulates the activation of alveolar macrophages, limiting macrophages' classical activation induced by IFN-γ, and promoting macrophages' alternative activation and proliferation in the presence of IL-4.

IV. INTRODUCTION

1.- The respiratory system

Respiration allows oxygen uptake, vital to generate the chemical energy, and a concomitant removal of carbon dioxide and other gaseous metabolic wastes.

Anatomically, the respiratory system is divided into the upper and lower respiratory tracts. The upper tract structures consist of the nose, pharynx, and larynx, whilst the lower tract structures include the trachea, bronchi, bronchioles, alveolar ducts, and alveoli. Functionally, the same structures are also classified into the conducting zone and respiratory zone. The conducting zone consists of the nose, pharynx, larynx, trachea, bronchi, and bronchioles that form a continuous passageway for air to move in and out of the lungs. Alternatively, the respiratory zone is found deep inside the lungs and is made up of the respiratory bronchioles, alveolar ducts, and alveoli [18] (Fig. 1).

The alveoli are the main structure involved in gas exchange and their walls are composed of three layers [18] (Fig. 1):

- *The capillary endothelium* is composed of endothelial cells, which line the inner surface of the pulmonary capillaries and form an interface between bloodstream and alveoli facilitating gas exchange and metabolic functions.
- *The interstitial layer* lies between the capillary endothelium and the alveolar epithelium and is comprised of collagen and elastin fibbers embedded in a hydrated porous proteoglycan gel. These components provide support and elasticity to the alveolar units. The extracellular matrix also contains fibroblasts, mast cells, pericytes and interstitial macrophages.
- *The alveolar epithelium* is a physical and functional barrier, which is also involved in the clearance of environmental agents. Two types of epithelial cells form the alveolar epithelium [19, 20]:
    - *Type I pneumocytes* are flattened cells that constitute 40% of alveolar lining cells but cover ~90% of the alveolar surface facilitating gas exchange.
    - *Type II pneumocytes* are cuboidal cells (with apical microvilli) that perform essential functions to maintain alveolar homeostasis. Firstly, they are responsible for the synthesis, secretion and recycling of

pulmonary surfactant. Additionally, they participate in epithelial repair and play an important role in the immune defence of the lungs.

The epithelial walls of the alveoli are covered by the alveolar fluid, which contains immune cells (mainly resident alveolar macrophages at the steady-state), as well as soluble proteins and peptides, and pulmonary surfactant [21].

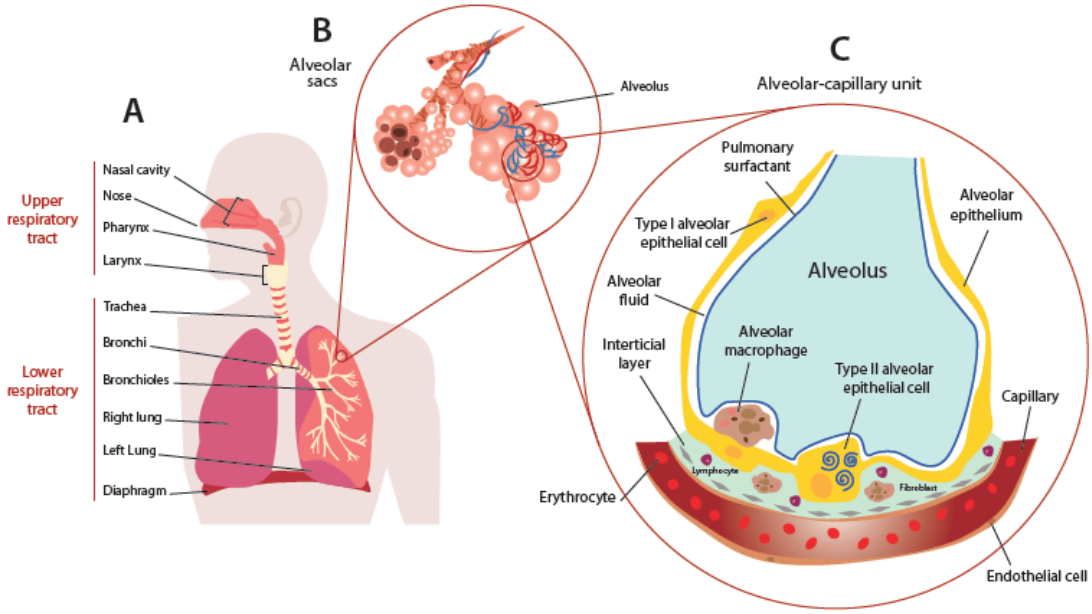


Figure 1. A) Schematic representation of the human respiratory system. B) Grouped alveoli forming two alveolar sacs. C) Schematic representation of the alveolar-capillary, the gas-exchange unit, showing their three layers and their components: alveolar epithelium, interstitial layer and capillary endothelium.

2.- Mucosal immunity in the respiratory tract

Mucosal surfaces comprising the respiratory tract, the gastrointestinal tract, and the urogenital tract, represent the most important portal of entry for pathogens, especially bacteria and viruses. Pathogens may either replicate and promote disease at the initial mucosal site or invade neighbouring tissues and the blood stream, inducing disease at distant systemic localities [22].

In particular, the lung has the largest area of the body in contact with the external environment. It is estimated that there are about 300 million alveoli in the adult human lungs, representing an area of contact with the outside 60 times larger than the surface of the skin. This massive area of contact with the external environment facilitates oxygenation but increases the risk of infection and inflammation by pathogens and endotoxins present in the air we breathe. Therefore, airway mucosal surfaces must employ robust non-specific as well as specific mechanisms to be protected from respiratory tract infections [21, 23].

Mechanical defences prevent particulate antigens and microorganisms from entering the lungs. These mechanisms begin at the nose, which functions as a filter by capturing or trapping large particles in the nasal hair or fimbriae [24]. The smaller particles that pass this filter are then inhaled and deposited in the lower airways, where mucins of the mucociliary blanket lining the airways surface act by trapping and removing them through ciliary movements [25]. The particles or microorganisms that pass this barrier are carried directly to the alveolar space, where they encounter the components of the alveolar innate immune system [26, 27].

3 - Components of the innate immune system in the alveolus.

The innate immune system in the alveolar space can be divided into the humoral component, which comprises ubiquitous and secreted antimicrobial peptides and proteins (lactoferrin, lysozyme, pulmonary collectins (SP-A and SP-D), β-defensins and other antimicrobial peptides) able to neutralize and eliminate microbial components and pathogens, and the cellular component, composed of resident and recruited specific cells that participate actively in host defence [21].

3.1 - The cellular component: Lung Macrophages

In addition to the vast repertoire of molecules that comprise the humoral innate defence, the innate system has a cellular arm that is formed by alveolar epithelial cells, macrophages, dendritic cells, innate lymphoid cells, neutrophils, eosinophils and other granulocytes. Their abundance and importance varies depending on many factors such as development and health conditions [19].

At least three types of macrophages reside in the lungs: bronchial macrophages, interstitial macrophages and alveolar macrophages. Moreover, humans and other mammals (but no rodents) present intravascular macrophages located on the

inner side of capillaries [28]. Alveolar macrophages are found in the air space of the alveoli, where they comprise 90–95% of the cellular content in the steady state [2]. It has been reported that only a single alveolar macrophage is detected in approximately three alveoli [29]. However, it is thought that alveolar macrophages may travel between alveoli through the connecting pores of Kohn patrolling the entire lung surface [30]. Interstitial macrophages are located in the parenchymal space (interstitium) between adjacent alveoli, where they interact with dendritic cells and interstitial lymphocytes [2]. It has been proposed that interstitial macrophages promote immunity by presenting antigen to interstitial T cells. However, in a mouse asthma model it has been shown that they also promote tolerance and prevent T_H2-type airway inflammation by IL-10-mediated inhibition of DC activation [31]. By flow cytometric analysis, lung macrophages can be characterized by their expression of the surface macrophage markers as shown in table 1 [1, 2]:

Table 1. Surface molecule expression on lung macrophages. Expression of characteristic surface molecules on alveolar (AMs) and interstitial (IMs) macrophages, from high (+++) to low (+) to absent (–). '?' Indicates 'still unclear'. (Taken from [1, 2]).

Molecule	AMs	IMs
CD103	+	+
SIRPα	+	+
CD64	+	?
MerTK	++	+
Siglec F	+++	-
CD24	+	?
CD11c	+++	+
Ly6C	+	+
F4/80	+++	++
MHC class II	+	variable
CD11b	+	+
CD68	+	?
CD200R	++	?
CD206	++	+

3.1.1 - Alveolar macrophages

Because the lungs function as the body's gas-exchange organ, they are inevitably exposed to air that is contaminated with pathogens, allergens and pollutants. Host-defence mechanisms within the lungs must facilitate clearance of inhaled pathogens and particles while minimizing an inflammatory response that could damage the thin, delicate gas-exchanging epithelium [11]. For the naive host, the primary defences in the alveolar fluid are the resident alveolar macrophages and these cells function to maintain a tolerant state towards innocuous antigens present in the inhaled air [11].

Alveolar macrophages promote an immunologically tolerant state by balancing the responses of alveolar epithelial cells, dendritic cells and T cells to environmental challenges (Fig. 2). Previous studies established that alveolar macrophages suppress immune responses through the inhibition of dendritic cell-mediated activation of T cells and production of transforming growth factor-β (TGF-β) [32-34]. It is well established that TGF-β-induces forkhead box $P3^+$ ($Foxp3^+$) T regulatory cells. These cells generally suppress induction and proliferation of effector T cells [35, 36]. Alveolar macrophages produce TGF-β and retinoic acid that induce the generation of $Foxp3^+$ T regulatory cells from naive $CD4^+$ T cells, which induce tolerance to inhaled innocuous antigens [37]. In addition, TGF- β and prostaglandins produced by alveolar macrophages inhibit T cell activation [1]. Alveolar macrophages lose the ability to induce T regulatory cells and tolerance when harmless antigens are mixed with Toll-like receptor 4 ligands. The switch from a tolerogenic mode to an inflammatory mode is accompanied by induction of the secretion of IL-1, IL-6, TNF-α and other pro-inflammatory mediators [37].

Previous studies have indicated that the interaction between alveolar macrophages and epithelial cells also contributes to maintain tolerance. Specifically it has been suggested that detachment of alveolar macrophages from epithelia upon infection may unleash alveolar macrophage inflammation by withdrawal of active TGF-β [38-40]. It has also been published that inhibition of pathological airway inflammation occurs via the intercommunication of sessile alveolar macrophages located in different alveoli through the alveolar epithelium. A subset of alveolar macrophages form gap junctions with alveolar epithelial cells through the use of connexin Cx43 hemichannels that induce cyclic and synchronized waves of calcium release from both alveolar macrophages and alveolar epithelial cells. The communication between alveolar macrophages over distance via the epithelium, through synchronous Ca^{2+} spikes, is

immunosuppressive and mediates protection to the host from inflammatory acute lung injury following TLR ligation [29]. The prevention of inflammatory responses is also mediated by various inhibitory receptors on alveolar macrophages, with the ligands expressed on alveolar epithelial cells or present in the alveolar fluid. The interaction of the ligand CD200L on alveolar epithelial cells with its receptor CD200R on alveolar macrophages negatively regulates inflammatory responses induced by TLR signalling on alveolar macrophages [41]. Likewise, interaction of the signal-regulatory protein SIRPα (which mediates a so-called 'do not eat me' signal) on alveolar macrophages with the globular heads of the surfactant proteins SP-A and SP-D suppresses alveolar macrophages pro-inflammatory responses and phagocytosis, which can be overcome by TLR4 signalling that downregulates SIRPα [42]. Another class of immune-regulatory receptors that is expressed by macrophages is the triggering receptor expressed on myeloid cells (TREM) family. TREM-1-deficient mice efficiently control acute pulmonary bacterial and viral infection and undergo less morbidity than wild-type mice after infection with influenza virus, which indicates an anti-inflammatory role rather than a pro-inflammatory role for TREM-1. Conversely, silencing of TREM-2 causes an enhanced alveolar macrophage response to TLR4 agonists, which further emphasizes its role as a negative regulator of macrophage function. Alveolar macrophages upregulate TREM-1 expression in response to the recognition of TLR ligands, whereas TREM-2 is expressed by mouse alveolar macrophages following inflammation [1, 2].

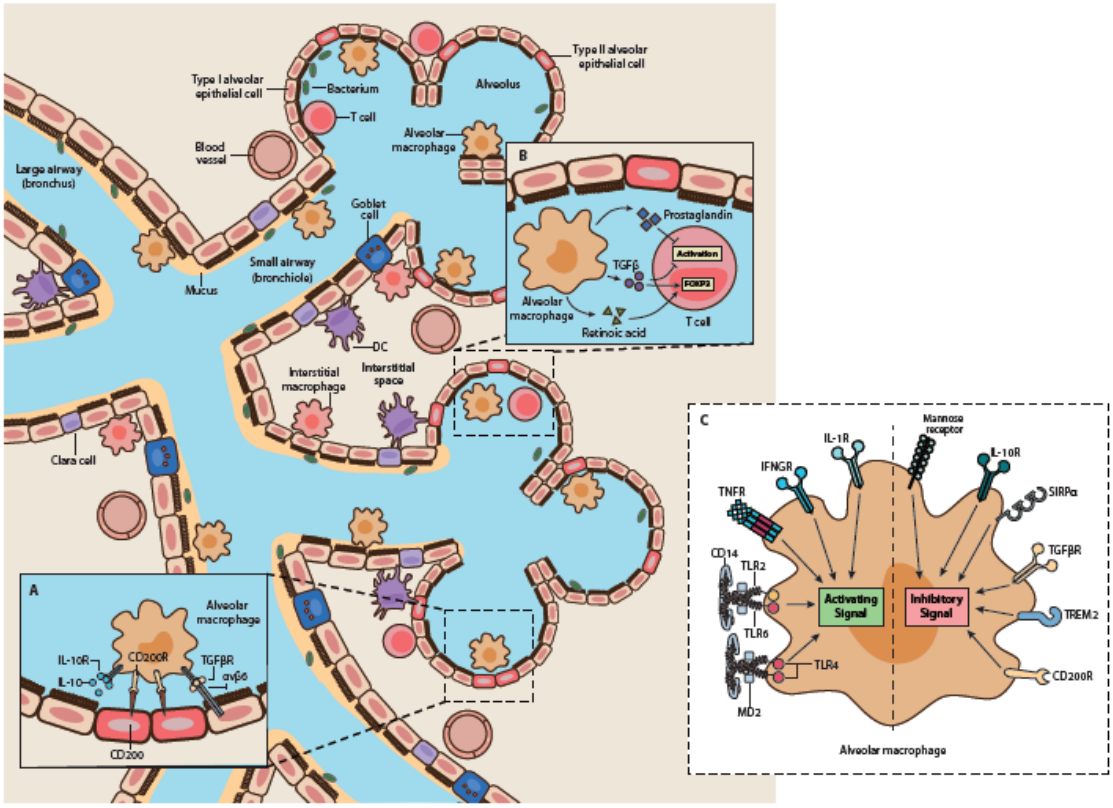


Figure 2. Leukocyte interactions in the healthy lungs. Alveolar macrophages inhabit in the alveolar lumen in contact with type I alveolar epithelial cells (which account for as much as 98% of the total surface area of the lungs) or with type II alveolar epithelial cells. Macrophages found in the bronchus (larger airways) reside within the mucous layer. Mucus-producing goblet cells are present in both bronchus (large airways) and bronchioles (small airways), and secretory non-ciliated Club cells are more common in the bronchioles. Macrophages are also located in the interstitial space between the alveoli and the blood vessels where T cells, dendritic cells (DCs) and a scarce population of B cells also reside. Commensal (and pathogenic) bacteria can reach the airway mucosa the alveolar surface. A) Alveolar macrophages are regulated by the airway epithelium through their interactions with CD200, which is expressed by type II alveolar cells, with transforming growth factor-β (TGFβ), which is tethered to the epithelial cell surface by αvβ6 integrin, and with secreted interleukin-10 (IL-10). These interactions can also take place in the larger airways, where CD200 and αvβ6 integrin are also expressed by the bronchial epithelium. B) The secretion of TGFβ and retinoic acid by alveolar macrophages can induce forkhead box P3 (FOXP3) expression in both naive and activated CD4+ T cells that are present in the lumen of the airways. In addition, TGFβ and prostaglandins suppress T cell activation. C) Alveolar macrophage activation and the initiation of inflammation involves a complex balancing act between activating and repressing signals. On the one hand, Toll-like receptors (TLRs), along with their co-receptors such as MD2 and CD14, recognize pathogen-associated molecular patterns to trigger inflammatory cytokines, such as tumour necrosis factor (TNF), interleukin-1β (IL-1β) and interferon-γ, which, through binding to their receptors, perpetuate inflammation. On the other hand, mediators such as IL-10 and soluble or αvβ6 integrin-tethered transforming growth factor-β (TGFβ) block pathways that induce inflammation. Cell-cell interactions with bronchial or alveolar epithelial cells also trigger inhibitory signals to alveolar macrophages, for instance, through CD200 receptor (CD200R), triggering receptor expressed by myeloid cells 2

(Trem-2) or signal-regulatory protein-α (SIRPα). Loss of the ligands for the negative regulators, for example, following epithelial cell loss during inflammation, will tip the balance towards alveolar macrophage activation. Conversely, increased expression of the negative regulators and inhibition of TLR signalling pathways, for example, during the resolution of inflammation, tips the balance towards the immunosuppression of alveolar macrophages. Taken from [1].

The negative signals of the inhibitory receptors are overridden by infection with a pathogen that unleashes the alveolar macrophages by combined ligation of several pathogen-recognition receptors of the TLR family, C-type lectin family, NLRP family and/or scavenger receptor family [43]. Alveolar macrophages are ideally suited to act as first line of innate cellular defence in the lower airways due to their localization in the alveolar lumen, where they are attached to alveolar epithelial cells and sample the microbes that are transported towards them by alveolar liquid flow [29].

It is well known that alveolar macrophages play a key role in the defence against bacterial, fungal and viral infections and in the prevention of acute lung injury through the limitation of and restoration of normalcy after infection-mediated damage. By virtue of their potent phagocytic ability, alveolar macrophages are essential for the clearance of pulmonary infection with bacteria and fungi, including *Streptococcus pneumonia, Mycobacteria tuberculosis, Pseudomonas aeruginosa and Pneumocystis carinii* [44-47].

3.1.2 - The development of alveolar macrophages

Various studies addressing the ontogeny of tissue macrophages have laid the basis for a change in the long-held dogma of mononuclear phagocyte development, which assumed that bone marrow-derived circulating blood monocytes constantly enter tissues to replenish resident macrophages [2]. It is now well accepted that many tissue macrophage subsets, including lung alveolar macrophages, arise from embryonic progenitors that seed the organs and mature locally before and shortly after birth. They are maintained by proliferative self-renewal throughout life independently of bone marrow–derived monocytes in the steady state [48, 49].

Like many other organs, foetal lungs contain two distinct myeloid populations, foetal macrophages and foetal monocytes, which are considered to derive from embryonic haematopoiesis in the yolk sac and foetal liver, respectively [50, 51]. Foetal macrophages that are generated during primitive haematopoiesis in the yolk sac appear around embryonic day 8.5 (E8.5) and subsequently colonize tissues via the

blood circulation [52]. Foetal monocytes are generated in the foetal liver around E12 and subsequently migrate to embryonic tissues, where they appear around E14 [53]. Both of those populations can be clearly identified in foetal lungs at E14.5–E18.5 [54, 55].

While the number of lung foetal macrophages remains relatively constant between E17 until around 1 week after birth, foetal lung monocyte populations expand dramatically, accompanied by a profound change in expression of characteristic surface markers during this period. They successively increase their expression of CD11c, Siglec-F, F4/80 and CD64 and concomitantly downregulate their expression of Ly6C and CD11b until they reach the mature stage. To reach the mature stage, lung environment seems to be essential [56-58] and transcriptional activity of peroxisome proliferator-activated receptor γ (PPARγ), following stimulation with granulocyte-macrophage colony-stimulating factor (GM-CSF), as well as activity of the transcriptional repressor Bach2 have been found to be necessary for alveolar macrophages maturation. Foetal monocytes are able to develop to mature alveolar macrophages upon adoptive transfer into the lungs of newborn mice, but foetal macrophages are not [54, 55] (Fig. 3).

After perinatal development, mature alveolar macrophages are maintained by homeostatic proliferation [54, 59] and self-renew without substantial contribution by immigrating monocyte precursor cells, as supported by the intact alveolar macrophage population in leukemic patients with decreased or absent blood monocytes [60]. The proliferative capacity of mature alveolar macrophages was already reported 40 years ago [61, 62]. Today, compelling evidence from several studies using genetic fate-mapping approaches, parabiosis and partial-body irradiation has demonstrated that progenitor cells derived from hematopoietic stem cells in adults do not contribute much to alveolar macrophages under steady-state conditions [54, 59, 62, 63]. However, they have the ability to differentiate into functional alveolar macrophages under certain conditions, as shown by transfer of bone marrow hematopoietic stem cells into lethally irradiated recipients with ablated tissue macrophages [54, 59, 64]. Impaired self-renewal ability of alveolar macrophages due to aging or certain environmental cues might also result in the replenishment of alveolar macrophages by circulating blood monocytes [2].

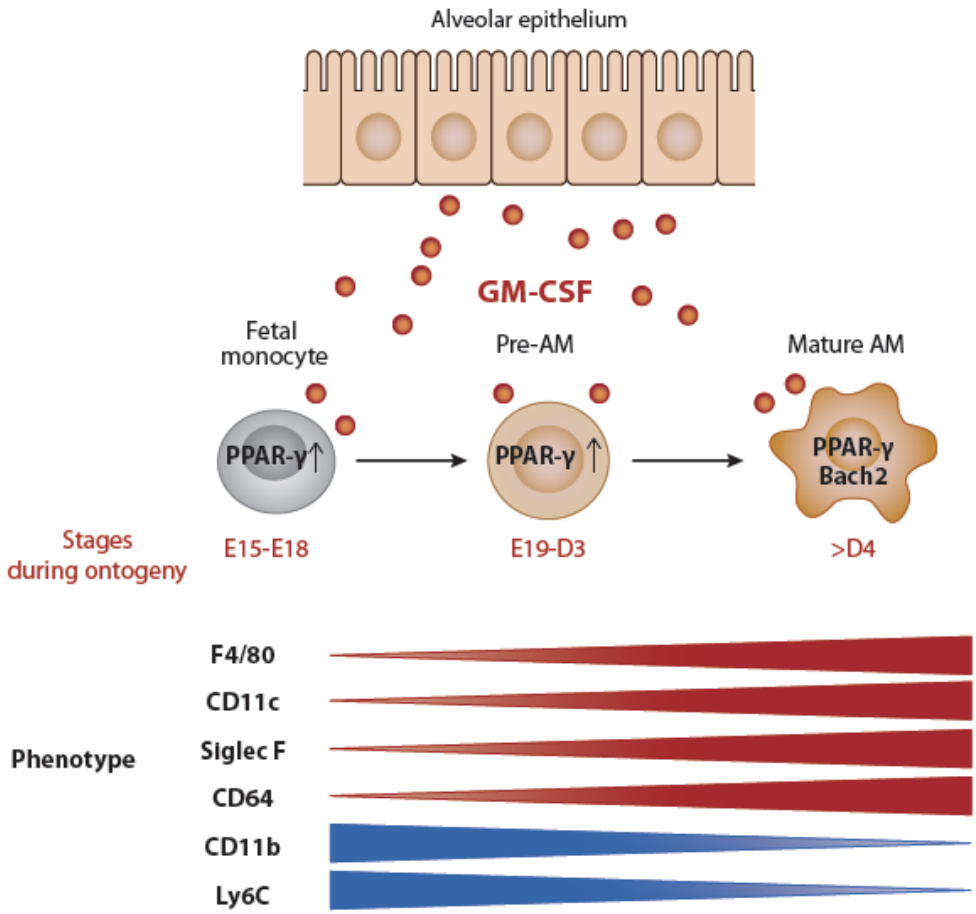


Figure 3. Development of alveolar macrophages. Foetal monocytes seed the lungs at around E15. The production of GM-CSF (CSF2) by alveolar epithelial cells induces expression of PPAR-γ in foetal monocytes, which 'instructs' the development and function of alveolar macrophages. There is gradual up- and downregulation of cell surface markers during AM ontogeny. E15, E18 and E19, embryonic day 15, 18 and 19. D3 and D4, day 3 and day 4 after birth. Taken from [2].

In the absence of GM-CSF, differentiation of the alveolar macrophage progenitor is already abrogated in the foetal lungs at around E17, which indicates that GM-CSF is instrumental for licensing alveolar macrophage development. In the mouse foetus, the highest GM-CSF expression is found in the lungs, where stromal cells are the main source that provides it in a paracrine manner to foetal monocytes [54, 55]. GM-CSF instructs lung foetal monocyte differentiation shortly before and after birth through activation of the nuclear receptor PPAR-γ, which controls the transcriptional program intrinsically required for the differentiation and function of alveolar

macrophages, including cholesterol metabolism, β-oxidation of fatty acids, lipid transport, storage and degradation [55].

Since alveolar macrophages participate in surfactant degradation and therefore the maintenance of lung surfactant homeostasis, disruption of GM-CSF signalling or the absence of alveolar macrophages results in the development of pulmonary alveolar proteinosis [65, 66]. Pulmonary alveolar proteinosis is a condition on which lungs suffer a progressive surfactant accumulation in alveoli that can lead to hypoxemic respiratory failure [67]. The development of pulmonary alveolar proteinosis in *Csf2*$^{-/-}$ mice and *Csf2rb*$^{-/-}$ mice provided first evidence of a critical role for this cytokine in lung homeostasis [67]. Another report confirmed that patients with acquired or congenital pulmonary alveolar proteinosis presented either auto-antibodies to GM-CSF or mutations in the genes encoding the α-chain or the β-chain of the GM-CSF receptor (GM-CSFR) [65]. Additionally, several reports have demonstrated, that *Csf2*$^{-/-}$ and *Csf2rb*$^{-/-}$ mice are completely devoid of alveolar macrophages [54, 59, 64]. These observations have established a link between GM-CSF and the development of functional alveolar macrophages capable to degrade pulmonary surfactant [65, 66].

3.1.3 - Activation of macrophages

Upon infection alveolar macrophages release cytokines that affect the activity or maturation of dendritic cells, which are believed to be the most potent antigen presenting cells. Dendritic cells are localized at the epithelial border where they capture antigens. Subsequently, they migrate to local draining lymph nodes and activate naïve T cells, which initiate the adaptive immune response [68-70].

After being primed by antigen presentation T- lymphocytes migrate from the lymph nodes to peripheral blood for subsequent extravasation at mucosal effector sites. Antigen-specific T cells, together with Innate lymphoid cells, are key effectors of immune functions, through the secretion of T_H1 and T_H2 type of cytokines among others [68-70].

The establishment of acquired immunity also triggers subsequent interactions between macrophages and activated T and B-lymphocytes providing macrophages a new level of regulation and acquisition of effector functions. It has been shown that T_H1-derived interferon-gamma (IFN-γ) is essential during cell-mediated immunity to

intracellular infection whereas T_H2-derived interleukin-4 (IL-4) is critical to fight extracellular parasitic infection [3, 4]. The fact that IFN-γ and IL-4 elicit distinct activation programs on cultured macrophages gave rise to the classification, as for the lymphocyte system (T_H1 vs. T_H2), of classical (M1 or M(LPS/IFN- γ)) and alternative (M2 or M(IL-4)) activation of macrophages [3, 4] (Fig. 4). However, *in vivo* under various physiological conditions, coexistence of macrophages in different activation states and unique or mixed phenotypes have been observed, a reflection of the diversity and plasticity of the monocyte-macrophage lineage, most likely caused by complex tissue-derived signals [71]. Therefore, M1 or M(LPS/IFN- γ) and M2 or M(IL-4) most likely represent extremes of *in vitro* generated macrophage phenotypes; whereas LPS/IFN-γ and IL-4 in combination with tissue-specific signals would elicit more "modular" phenotypes during *in vivo* activation of macrophages.

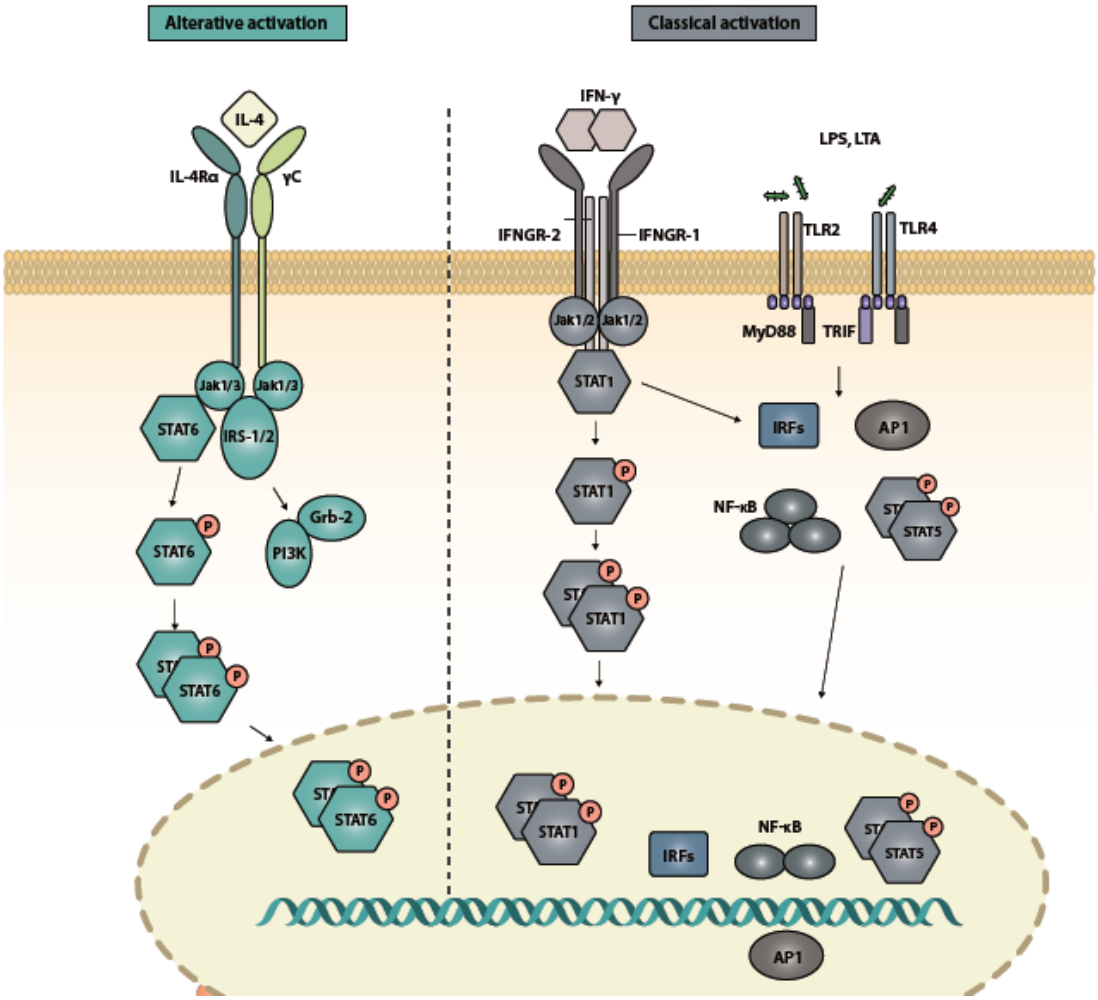


Figure 4. The signalling behind the effects of classic and alternative stimuli in macrophages. Alternative activation is mainly induced by IL-4 binding to its receptor, the heterodimer formed by IL4Rα and the common γ chain (γc). This triggers Jak1/3 driven phosphorylation of STAT-6, which dimerizes and translocates to the nucleus to activate gene transcription. IRS-1/2 are also phosphorylated and activate PI3K and the adaptor Grb2. Classic activation is elicited by stimulation with both IFNγ and lipopolysaccharide (LPS) or lipoteicoic acid (LTA). IFNγ binds to the heterodimeric receptor IFNGR1/2 to activate Jak1/2 and elicit phosphorylation and dimerization of STAT1 that translocates to the nucleus and activate transcription of pro-inflammatory genes. LTA and LPS bind to their receptors Toll like receptor 2 and 4 (TLR-2 and -4), respectively, which trough the adaptor proteins MyD88 and TRIF lead to activation of the transcription factors AP-1, NF-κB and STAT5. Also, Interferon regulatory factors (IRFs) are activated upon IFNγ, LPS and LTA binding to their receptors. Taken from [4].

3.1.3.1 - Classical activation of macrophages

The so-called classical stimuli are grouped according to their ability to induce prototypic inflammatory responses and markers, but their source, role, receptors, and signalling pathways differ substantially [3, 4]. IFN-γ is the main cytokine associated with classical activation of macrophages and the main T_H1 cell product. Other cells, such as natural killer (NK) cells and macrophages, themselves have been shown to produce this cytokine.

The IFNGR-1 and IFNGR-2 chains form the IFN-γ receptor complex, whose cytoplasmic tails are associated with Janus kinases 1 and 2 (JAK1 and JAK2), respectively. IFN-γ binds to IFNγR1 with high affinity and activates crosslinking of two molecules of each IFNγR1 and IFNγR2 chain, which results in activation of tyrosine kinases Jak1 and Jak2 and phosphorylation of STAT1 (signal transducers and activators of transcription 1). Activated STAT-1 translocates to the nucleus, where it mediates the transcription of IFN-γ-induced genes such as C-X-C motif chemokine 10 (CXCL10) or Interferon regulatory factors (IRFs), among others [72-74]. IFN-γ controls specific gene expression programs involving cytokine receptors, cell activation markers, and a number of cell adhesion molecules [75].

IFN-γ and IFN-γ receptor-deficient mice are viable and fertile and their steady-state macrophage populations appear normal [76, 77]. In these mice, however, macrophages show impaired production of antimicrobial products, and consequently, mice are susceptible to *Mycobacterium bovis* and *Listeria monocytogenes* infection. This defect is not only important for prototypical type-1 immune responses against bacteria and virus: IFN-γ deficient mice are susceptible to protozoa, such as *Trypanosoma cruzi* [78], *Leishmania amazonensis*, *Leishmania major* [79], and *Cryptosporidium parvum* [80], as well as showing a compromised defence against some nematodes (e.g. *Litomosoides sigmodontis* [81], *Schistosoma mansoni* [82], and *Schistosoma japonicum* [83]). In humans, mutations resulting in the lack of expression of the receptor lead to severe immunodeficiency (e.g. susceptibility to mycobacteria *M. avium; M. kansasii* and *M. chelonei*) [84].

Frequently IFN-γ is used in combination with Toll like receptor (TLR) ligands to induce classical activation of macrophages [85, 86]. Entire bacteria induce gene programs similar to those of isolated Toll-like receptors (TLRs) ligands, and major parts of the pathogen profiles can be ascribed to TLR ligands, such as LPS, muramyl

dipeptide, and lipoteichoic acid [86]. LPS is the best-studied classical-activation of macrophages signal and is recognized by TLR4, although recent evidence shows that LPS can also be recognized by TLR4-independent mechanisms leading to inflammasome activation [87, 88]. Conventionally, TLR4 activation induces myeloid differentiation primary response gene 88 (MyD88) and MaL/Tirap (MyD88 adapter like/Toll-interleukin 1 receptor domain containing adaptor protein)-dependent pathways that lead to strong pro-inflammatory cytokine profiles (e.g. IFN-β, IL-12, TNF-α, IL-6, and IL-1β), chemokines (e.g. CCL2, CXCL10, and CXCL11), and antigen presentation molecules, such as MHC members, co-stimulatory molecules, and antigen-processing peptidases. These pro-inflammatory profiles are controlled by nuclear factor of kappa light polypeptide gene enhancer (NF-κB), activator protein 1 (AP-1), IRFs, STAT1, and early growth response (EGR) family members, many of which participate in the IFN response. Although there is a degree of overlap between LPS and IFN-γ gene profiles, similarities are not enough to consider the stimuli to be homologous [72]. As for IFN-γ, the numbers of macrophages in TLR-deficient animals are normal, but their activation is defective and therefore survival to infection is severely impaired [89, 90]. In humans, genetic mutations in the TLR signalling pathway have been described, and as for mice, there is evidence for susceptibility to infection with mycobacteria, pneumococci, meningococci, malaria, and susceptibility to develop bacteremia [91].

Synergy between IFNγ and LPS to induce pro-inflammatory activation of macrophages has been extensively addressed. There have been described several mechanisms through which this synergy takes place: increase in TLR expression induced by IFNγ, synergistic activation of promoters by STAT1 and NF-κB, enhanced activation of NF-κB, additive functions of NF-κB/MAPK and STAT-1 induced genes, and IFN-γ-mediated suppression of feedback inhibition loops in LPS-signalling [92, 93]. Physiologically, the synergistic induction of pro-inflammatory chemokines and cytokines by IFNγ and LPS leads to the recruitment and activation of neutrophils and the induction of effector molecules (e.g. nitric oxide), which mediate the immune response against bacteria and intracellular parasites. In addition, pro-inflammatory cytokines can have multiple functions in multiple locations such as neuronal reaction, infectious agents generating pathological pain and regulation of apoptosis in the infected cells [4].

A common *in vitro* model for the study of classic vs. alternative activation of human macrophages is GM-CSF- and macrophage colony stimulating factor (M-CSF)-generated macrophages from peripheral blood monocytes. It has been determined that

some GM-CSF-mediated regulators are common with the IFN-γ and TLR signalling pathways [94] and GM-CSF is considered to drive macrophages to a classically activated phenotype [95]. *In vitro* experiments on GM-CSF-treated monocytes indicated that GM-CSF promoted the acquisition of pro-inflammatory markers (INHBA, MMP12, EGLN3, CCL17, ECSCR, and CCR2) [96-98]. Furthermore, GM-CSF enhances antigen presentation, complement- and antibody-mediated phagocytosis, microbicidal capacity, leukocyte chemotaxis, and adhesion. GM-CSF induces monocyte and macrophage cytokine production of IL-6, IL-8, G-CSF, M-CSF, TNF-α, and IL-1β, but less than, for example, LPS. Other stimuli that share pro-inflammatory properties have been termed classical activation inducers (e.g. TNF, IL-1β, and IL-6) [99].

3.1.3.2 - Alternative activation of macrophages

The alternative stimuli are commonly grouped by their ability to antagonize prototypic inflammatory responses and markers; however, as for M1 stimuli, their source, role, receptors, and signalling pathways differ [4]. One of the main factors that induce alternative activation is interleukin-4 (IL-4). IL-4 is produced by the T_H2 cells, eosinophils, basophils, or macrophages themselves and is recognized by two different receptor pairs. IL-4Rα can pair with the common gamma chain (γc), enabling IL-4 binding, and with the IL13Rα1 chain, enabling IL-4 or IL-13 binding. Cytoplasmic tails of the transmembrane receptors IL-4Rα, γ-c and IL-13Rα1 are bound to JAKs. Upon ligand-induced dimerization of the receptor complex JAKs become trans- and auto-phosphorylated [100]. The activated JAKs phosphorylates tyrosine residues in the cytoplasmic tails of the receptor, which then serves as docking sites for a number of adaptor or signalling molecules including STAT6. JAK1 phosphorylates STAT6, which then dimerizes and translocates to the nucleus. Other transcription factors activated upon IL-4 stimulation of macrophages include c-Myc, IRF4, and Krüppel-like factor 4 (KLF4). IL-4 induces macrophage fusion and decreases phagocytosis. The IL-4-induced transcriptome in human and mouse includes transglutaminase 2 (TGM2), mannose receptor (MRC1), cholesterol hydroxylase CH25H, and the prostaglandin-endoperoxide synthase PTGS1 (prostaglandin G/H synthase 1), and the signalling modulators CISH and SOCS1 [101]. However, in murine macrophages typical IL-4Rα-mediated activation markers include: Resistin-like molecule alpha (RELMα), Ym1, Arginase-1 and Mannose Receptor (CD206) [73].

Insulin receptor substrate 1 and 2 (IRS1 and IRS2), members of the insulin receptor substrate family are also recruited to the IL-4 receptor complexes. Phosphorylated IRS

activates PI3K and the adaptor Grb2. Recently, it has been shown that IL-4 induces macrophage proliferation [5, 102] through activation of the PI3K/Akt signalling pathway [103]. In IL-4-deficient animals, the numbers of macrophages and maturation are normal, and defects appear in the immune response against nematodes and some viral infections [3, 104]. In humans, polymorphisms in the IL-4R have been associated with the development of asthma and atopy [105, 106].

As discussed before GM-CSF and M-CSF are frequently used for *in vitro* model for the study of classic vs. alternative activation, respectively, of peripheral blood monocytes-derived human macrophages. M-CSF binding leads to receptor dimerization, auto phosphorylation, activation of ERK, phosphatidylinositol 3-kinase, phospholipase C, and eventually Sp1 transcription factor nuclear localization [85]. The transcriptional response to M-CSF includes SERPINB2, folate receptor β (FRβ), encoded by the *FOLR2* gene [97, 98], transient gene clusters with overrepresentation of cell cycle genes (e.g. cyclins A2, B1, D1, and E1) and downregulation of human leukocyte antigen (HLA) members and stable gene clusters, including TLR7 and the complement C1QA/B/C subunits [85].

Other stimuli that drive “alternative” or anti-inflammatory/regulatory activation of macrophages are: IgGs, glucocorticoids, IL-10 and TGF-β [4].

3.1.3.3 - Classically and alternatively activated macrophages in disease

Following tissue injury or infection by viral or bacterial pathogens, the first-responder macrophages usually exhibit an inflammatory phenotype and secrete pro-inflammatory mediators such as TNF-α, nitric oxide and IL-1, which participate in the activation of various antimicrobial mechanisms, including oxidative processes that contribute to the killing of invading organisms [107, 108]. Other mediators produced by classically activated macrophages include IL-12 and IL-23, which are decisive in influencing the polarization of T_H1 and T_H17 cells, which further drive inflammatory responses forward [109]. Classically activated macrophages produce reactive oxygen intermediates, such as superoxide, and, in the case of murine macrophages, nitrogen intermediates, including NO that are highly toxic for microorganisms but can also be highly damaging to neighbouring tissues and lead to aberrant inflammation [110]. Indeed, classical macrophages are believed to participate in various chronic inflammatory and autoimmune diseases. Therefore, pro-inflammatory and antimicrobial

classical macrophage responses must be controlled to prevent extensive collateral tissue damage to the host [111].

A protective role in tumorigenesis has been described for classically activated macrophages, which activate tumour-killing mechanisms and antagonize the suppressive activities of tumour associated macrophages and other cells which have been shown to suppress adaptive tumour-specific immune responses and promote tumour growth, invasion, metastasis, stroma remodelling and angiogenesis [112-117]. Classic macrophages also amplify T_H1 responses, providing a positive feedback loop in the anti-tumour response [71].

By contrast, tumour associated macrophages isolated from solid and metastatic tumours have a suppressive alternative-like phenotype. Furthermore, accumulating evidence from many tumour models suggests that macrophages contribute to tumour progression, with increasing numbers of tumour associated macrophages among other cells [118-120]. These observations are also consistent with the tumour-promoting activities of IL-4 and IL-13, which also promote alternative macrophage differentiation [121-124].

In addition to their innate phagocytic activity and role in antimicrobial immunity, macrophages are intimately involved in wound repair [125-128]. In contrast to pro-inflammatory and anti-microbial classical macrophage responses, alternative macrophages exhibit potent anti-inflammatory activity and have important roles in wound healing, allergy, asthma and fibrosis [129-131]. The antagonism to classical macrophage responses elicited by alternatively activated macrophages, may be crucial for the activation of the wound healing response and for tissue homeostasis to be restored. It has also been shown that classically activated macrophages can themselves 'convert' into anti-inflammatory macrophages with an alternative wound-healing phenotype [71, 132]. To activate the wound healing response, alternative macrophages produce growth factors that stimulate differentiation and proliferation of epithelial cells and fibroblasts, including TGFβ1 and PDGF [133]. Macrophage-derived TGFβ1 contributes to tissue regeneration and wound repair by promoting fibroblast differentiation into myofibroblasts, by enhancing expression of tissue inhibitors of metalloproteinases that block the degradation of extracellular matrix and by directly stimulating the synthesis of interstitial fibrillar collagens in myofibroblasts [134, 135]. Macrophage-derived PDGF also stimulates the proliferation of activated extracellular matrix-producing myofibroblasts [136].

Alternatively activated macrophages can also regulate wound healing independently of their effects in myofibroblasts. Indeed, they produce matrix metalloproteinases and tissue inhibitors of metalloproteinases that control extracellular matrix turnover [137], engulf and digest dead cells, debris and various extracellular matrix components that would promote tissue-damaging classical macrophage responses [133, 138], and secrete specific chemokines that recruit fibroblasts, T_H2 cells and regulatory T cells [139, 140]. Moreover, alternatively activated macrophages produce factors that induce myofibroblast apoptosis [141], serve as antigen-presenting cells that propagate antigen-specific T_H2 and T regulatory cell responses, which promote wound healing while limiting the development of fibrosis [142, 143], and express immune-regulatory proteins (such as IL-10, RELMα, chitinase-like proteins and arginase 1) involved in promotion of wound healing and reduction of the magnitude and duration of inflammatory responses [144-149]. It has been suggested that a deregulation of the activity of alternatively activated macrophages can lead to the initiation and maintenance of fibrosis [109].

Numerous studies have identified roles for alternatively activated macrophages in allergic responses driven by IL-4 and IL-13. However, their function in allergy and asthma remains controversial, with some studies suggesting that M(IL-4) macrophages promote allergic inflammation and others indicating a suppressive role for these cells [109].

Alternatively activated macrophages are also involved in the development of T_H2-dependent immunity to some extracellular parasites and fungi [150]. Regarding their role during extracellular parasites infections, it has been reported that alternatively activated macrophages play a central role in nematode expulsion during intestinal infection, both in the memory response to a secondary infection with *Heligmosomoides polygyrus* [151], and in expulsion of primary *Nippostrongylus brasiliensis* infection [152]. In both these settings, parasite clearance is dependent upon a strong T_H2 response, which acts to rapidly recruit immune cells including macrophages to the infection site and to stimulate their expression of Arginase 1, RELMα, and Ym1/2 in a STAT-6-dependent manner [150].

Importantly, the classic dogma of macrophage differentiation from recruited monocytes during inflammation was challenged by a study that demonstrated that tissue macrophages undergo massive proliferation during nematode infection [5]. In this scenario, IL-4 produced by T_H2 cells is sufficient to cause local macrophage

proliferation, resulting in increased numbers of alternatively activated macrophages, which are essential for parasite expulsion and wound healing during helminth infection [5]. Furthermore, recruited classically activated macrophages were induced to proliferate as long as sufficient IL-4 was present [5]. The signalling mechanism regulated by IL-4 to push macrophages into the cell cycle seems to be dependent on Akt phosphorylation [103]. It has been hypothesized that the expansion of tissue macrophages by proliferation would bypass the requirement for bone marrow-generated monocytes and thus allows local sites to rapidly achieve macrophage-mediated effector functions [109].

Moreover, accumulating evidences suggest that the activity of macrophages has a key role in progression to obesity [153]. The activation program of macrophages in obese and nonobese individuals differs sharply [154]. Additionally, the number of macrophages embedded in adipose tissue rises as a person's body fat increases [155]. These classically activated or "M1" macrophages produce, among others, the pro-inflammatory cytokine TNF-α that act systemically and affect metabolism by decreasing the sensitivity of other cells to insulin [156]. Adipose tissue itself releases a collagenous protein hormone into the circulation called adiponectin that modulates a number of metabolic processes, including glucose regulation and fatty acid oxidation[153] . However, in obesity, adiponectin production declines and macrophages produce resistin, which induces insulin resistance and glucose intolerance leading to diabetes[153] . Slender individuals have fewer macrophages per gram of body fat and are in an alternatively activated or "M2" state [157]. This activation program is induced by IL-4 or IL-13, derived from fat-tissue residential eosinophils [158]. Downstream of the IL-4Rα, through which both IL-4 and IL-13 signal, is the nuclear hormone receptor PPARγ that, when activated by appropriate lipids, inhibits the expression of genes that promote inflammation and protects against insulin resistance [159, 160]. Accordingly, it has been shown that cell-intrinsic lysosomal lipolysis plays a critical role during alternative activation of macrophages, explaining why and how M2 polarization is dependent on fatty acid oxidation [161]. Whereas M2 macrophages are fuelled by oxidative phosphorylation and have increased levels of Arg-1, AMPK (5' AMP-activated protein kinase) and the liver type-phosphofructokinase 2 or 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 1 (PFKFB1) , M1 macrophages rely on glycolysis for ATP production and have increased levels of iNOS, HIF-1α (hypoxia-inducible factor 1-alpha) and ubiquitous PFK2 or PFKFB2 [162, 163].

Finally, The plasticity of alveolar macrophages to change from one activation state to another could be important to keep the alveoli sterile and not inflamed. Alteration or dysregulation of this balance M1↔M2 might be associated with respiratory diseases since the persistence of a pro-inflammatory response (M1) is harmful to the tissue [4, 7] and, conversely, the permanence of an alternatively activated phenotype (M2) favours bacterial and viral infections, promotes tumour progress and the development of certain chronic respiratory diseases such as pulmonary fibrosis [4, 8].

Alveolar macrophages are located in a unique tissue compartment. They are covered by an aqueous fluid enriched in a lipo-protean material called pulmonary surfactant, which is essential to keep the alveoli open during cycles of inhalation-exhalation [9]. Pulmonary surfactant not only protects the lung from alveolar collapse, but also participates in the innate immune defence [9], especially due to the activity of SP-A and SP-D [11]. The potential effect of surfactant components, in particular of SP-A, on alveolar macrophage polarisation towards a pro-inflammatory phenotype M1 or an anti-inflammatory phenotype M2 has not been investigated in depth.

3.2 - The humoral component of the innate immune defence

The particles or microorganisms that reach the alveolar spaces get in contact with a range of soluble mediators produced by cells of the respiratory tract and therefore present in the mucus. These molecules can lead to lysis of pathogens directly through direct antimicrobial activity, or indirectly through opsonisation of bacteria and virus or the recruitment of inflammatory cells [21]. Among others, the most abundant and important defence proteins in the alveolar fluid are: immunoglobulin G, lysozyme, lactoferrin, cathelicidins, defensins, pattern recognition receptors (i.e. pentraxins, LBP, soluble CD14), galectins and some components of the pulmonary surfactant [21, 164, 165] (Fig. 5).

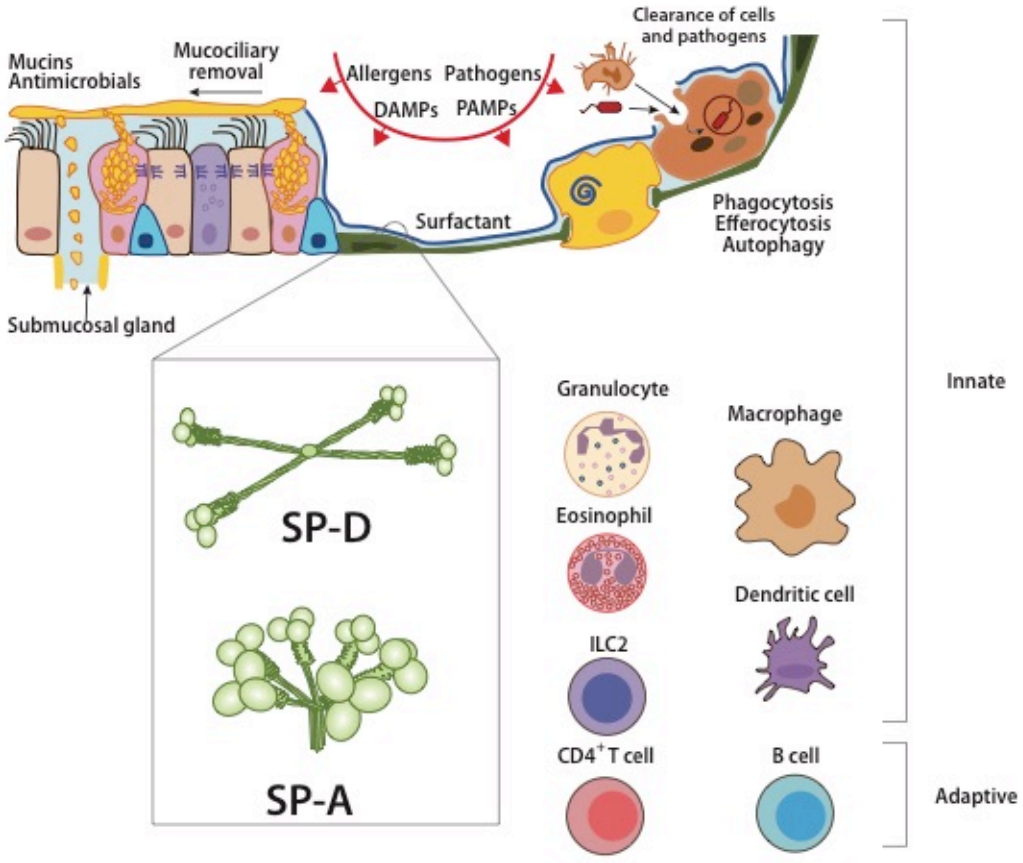


Figure 5. Integration of innate and adaptive components in the alveoli. PAMPs derived from commensal microbes, allergens or pollutants are recognized and cleared by innate immune components (i.e. alveolar macrophages, epithelial cells and surfactant collectins) to avoid unnecessary inflammation. Respiratory pathogens and lung damage, activate the cellular components of the innate immune system, which induce the production of cytokines, chemokines and antimicrobial proteins that recruit and activate cells of the innate and adaptive immune systems and regulate barrier function. Taken from [166].

3.2.1- Pulmonary surfactant

Pulmonary surfactant is present in the alveolar fluid as an extracellular membrane network that covers the air-liquid interface. Its main function is to reduce the surface tension at the air-liquid interface, thereby preventing alveolar collapse at the end of expiration [167]. Moreover, lung surfactant improves the mucociliary transport, and together with alveolar macrophages, constitutes the front line of defence against inhaled pathogens and toxins [168].

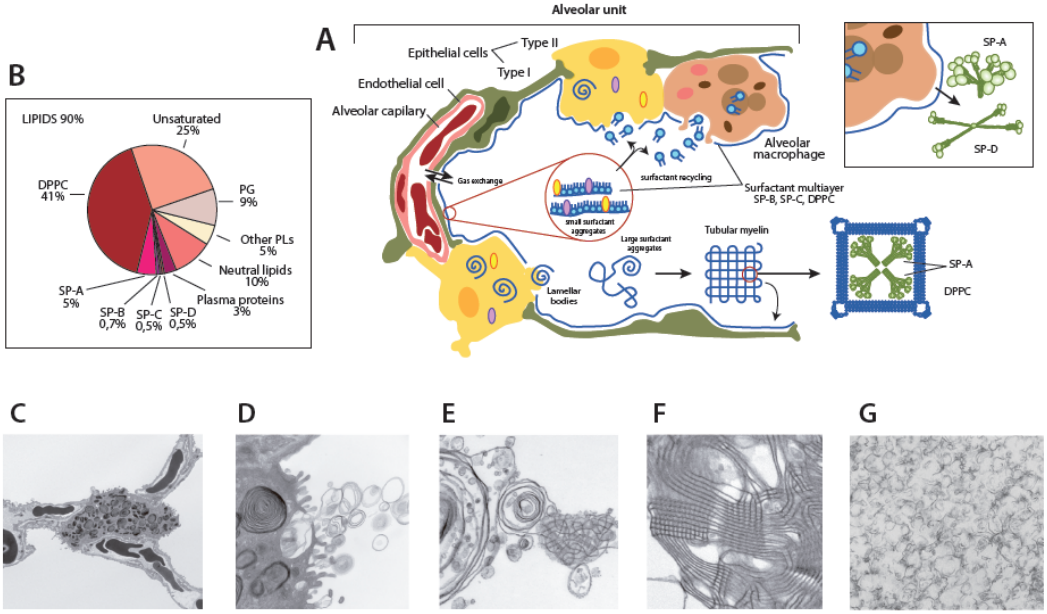


Figure 6. Integration of pulmonary surfactant function, secretion and recycling, and composition. After synthesis by type II epithelial cells (A and C), pulmonary surfactant (detailed composition in B) is stored as tightly packed bilayer membranes called lamellar bodies that are then secreted by exocytosis to the alveolar fluid (A and D), where spontaneously unravel in a lattice-like structure termed tubular myelin and large surfactant aggregates (A and E-F). They have high surface activity and adsorb very rapidly to the air-liquid interface preventing alveolar collapse at the end of expiration. These structures form a surface film at the alveolar air/liquid interface consisting of a phospholipid monolayer with bilayer structures attached to it. With surface compression and expansion cycles, small surfactant aggregates (A and G) with poor surface activity are generated, which are taken up and degraded by alveolar macrophages and recycled by type II cells. The biophysical activities of surfactant are integrated with alveolar host-defence functions that are mediated by the structural components of surfactant (SP-A, SP-B, SP-C and lipids) and soluble SP-D, which have intrinsic antimicrobial activity. Tubular myelin, formed by surfactant proteins SP-A and SP-B, and lipids create a highly structured reservoir of surfactant and host defence proteins that interact with alveolar macrophages and other cells of the immune system to bind to and remove microbial pathogens and 'instruct' inflammatory cells to mount appropriate host-defence responses (A). Taken from [9, 166]

3.2.2 - Surfactant protein A

SP-A represents approximately ~3-5% of the total mass of surfactant. It is synthesized and secreted by type II pneumocytes and nonciliated bronchial epithelial cells (Club cells, previously called Clara cells). This protein has ability to bind lipids, and therefore is tightly associated with surfactant membranes. SP-A participates actively in surfactant adsorption, recycling, and subsequent homeostasis [11, 169] (Fig. 6).

3.2.2.1 - SP-A structure

SP-A along with SP-D, belongs to the mammalian collectin family, which are secreted to the alveolar or other mucosal fluids [11]. Collectins are characterized by the presence of an N-terminal collagen-like region and a C-terminal lectin domain, comprising a Ca^{2+}-dependent carbohydrate recognition domain (CRD) [170]. Other members of the collectin family of proteins include: mannose-binding lectin (MBL), conglutinin, collectin liver 1 (CL-L1), collectin placenta 1 (CL-P1), collectin kidney 1 (CL-K1), collectin of 43 kDa (CL-43) and collectin of 46 kDa (CL-46). Apart from CL-L1 and CL-P1, all collectins are soluble and secreted proteins [171]. C1q is another soluble collagenous defence protein structurally homologous to SP-A and MBL and although it does have a collagen-like N-terminal triple helix it is not a collectin, as it does not have a lectin domain (CRD). Therefore, it has been used as a tool to explore the functionality of SP-A's collagen domain in various studies.

SP-A primary structure shows four structural domains and SP-A mature protein presents supratrimeric oligomerization, consisting of 18 monomers (Fig. 7). Each monomer (36 kDa) is composed by the following domains [172]:

- **N-terminal domain**

It is formed by 8-11 aminoacids, depending on species and variation in N-terminal processing. Two cysteines residues of this region (positions -1 and 6) have been shown to participate in intermolecular disulphide bond formation, which stabilizes the oligomer [173, 174]. The Cys6 forms an interchain disulphide bond that is necessary for the interaction of the protein with surfactant lipids and type II pneumocytes [175].

- **Collagen domain**

The collagen domain or collagen-like region of SP-A consists of 23 Gly-X-Y repeats, where X and Y are generally proline or hydroxyproline [170]. These repeats are interrupted close to the middle. The interruption introduces a link in the region that gives flexibility to the protein, allowing SP-A to fold in a bouquet-like structure. The collagen domain of SP-A undergoes N- or O post-translational glycosylations. Subsequently, three monomeric collagen helices are coiled to form a stable tensile domain that is relatively resistant to proteases [176]. This domain is thought to be involved in several functions of SP-A, including protein oligomerization, chemotaxis,

receptor-mediated effects, pathogen agglutination, increased phagocytosis, and binding to target ligands [17, 172, 177].

- **Neck domain**

This domain is localized between the collagen-like and globular domains and comprises a segment of 34 aminoacids with α-helical structure. It is directly involved in the trimerization of monomers, since three neck domains associate to form a triple coiled-coil structure, maintained by hydrophobic interactions. These structures have been also observed in other proteins of the collectin family such as SP-D or mannose-binding lectin [170]. In addition, this domain is critical for the selective binding of SP-A to various ligands, since it can guide the globular domains of SP-A trimers [176].

- **Globular domain**

The CRD of SP-A is composed of 123 aminoacids, which make up the C-terminal segment of the protein. This domain has 4 cysteines that form two intrachain disulphide bonds (Cys204-Cys218 and Cys135-Cys266). It also has 18 highly conserved residues, which are common among C-type lectins [178]. This domain resembles a globe-like structure, which is suitable for the interaction of SP-A with a range of ligands and lipid membranes [179]. In this domain there are at least two calcium-binding sites [179, 180].

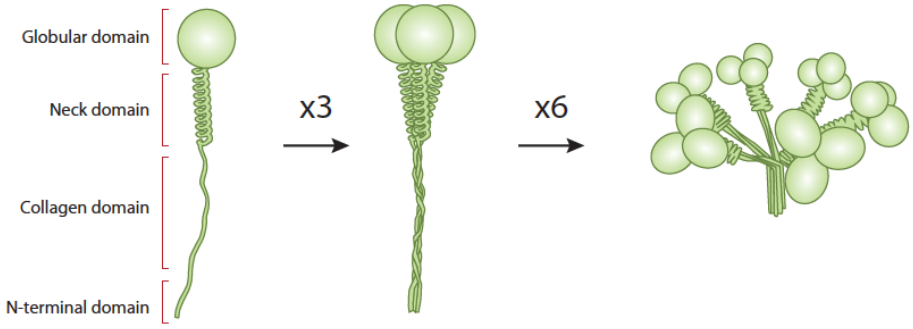


Figure 7. SP-A oligolimerization and structural domains. SP-A monomers consists of a globular domain, neck , collagen-like and N-terminal domains. Three monomers oligomerize to form a triple coiled-coil structure, leading to the formation of trimeric subunits. Trimeric subunits multimerize subsequently into octadecameric forms.

3.2.2.2 SP-A processing

SP-A is synthesized as a preprotein with a leader sequence of 17-28 amino acids, which is cleaved from the nascent peptide as it is translocated into the endoplasmic reticulum [181].

After synthesis, SP-A is extensively modified in the rough endoplasmic reticulum and Golgi [182]. Most of the folding and assembly of SP-A occurs before the protein exits the endoplasmic reticulum. SP-A is also co-translationally glycosylated with mannose-rich, endoglycosidase H sensitive carbohydrates in the endoplasmic reticulum. Late post-translational carbohydrate modifications in the Golgi apparatus include sialylation [183] and sulfation [184]. The complex folding, assembly and processing of SP-A likely contributes to the relatively slow rate at which SP-A traverses the secretory pathway [185].

SP-A is secreted into the alveolar space by two or more routes; via a regulated pathway that includes the lamellar body [186, 187] and a principal constitutive pathway that bypasses that compartment [188].

SP-A multimerizes in a process that can be conceptualized in two parts: First, SP-A trimers are built up by the association of three polypeptide chains. The collagen regions intertwine to form a collagen triple helix. Second, octadecamers are formed by lateral association of the N-terminal half of six triple-helical stems to yield the complete protein, an octadecamer of 650 kDa and 20 nm in length, which has the appearance of a bouquet of tulips [189, 190]. Furthermore, mammalian SP-A is not only assembled in supratrimeric oligomers but can also form multimers by self-association in the presence of Ca^{2+} [17]. Supratrimeric oligomerization and multimerization of SP-A are required for many of its functions, because the binding activity of a single SP-A lectin domain is very low [17, 173, 177]. Among carbohydrates, SP-A binds preferentially to mannose and fucose, which are sugars commonly found on fungal and microbial surfaces [191]. One of the most important properties of SP-A is its ability to bind lipids, preferentially to dipalmitoylphosphatidylcholine or sphingomyelin [192-194]. In fact, and SP-A co-isolates with surfactant lipids and is highly associated with them [194]. SP-A's ability to bind to surfactant lipids improves the biophysical activity of surfactant membranes, protects them from the inhibitory action of serum proteins, and allows SP-A to position and concentrate together with surfactant membranes at the front lines of defence against inhaled toxins or pathogens [9].

3.2.2.3 SP-A gene and differences between SP-A1 and SP-A2

In the course of evolution, the genetic complexity of SP-A gene has increased, particularly in the regulatory regions (i.e. promoter, untranslated regions). Most species have a single SP-A gene however; in humans and primates SP-A is encoded by two genes (*SFTPA1* and *SFTPA2*) [195]. Both genes are expressed in type II pneumocytes [196], but only SP-A2 gene is expressed in tracheal and bronchial submucosal gland cells [197, 198]. It has been proposed hat both gene products may be expressed in a 2:1 ratio (SP-A1:SP-A2) [199].

Qualitative differences between *in vitro*–expressed variants SP-A1 and SP-A2 include differences in their ability to enhance phagocytosis by alveolar macrophages [200, 201] and inhibit surfactant secretion by epithelial type II cells [202]. Differences in their aggregation, structural stability, oligomerization properties, sugar-binding characteristics, and ability to form phospholipids monolayers have also been observed [177, 202-204]. In most of these assays, SP-A2 was shown to be more active than SP-A1 indicating that some fundamental differences may exist between SP-A1 and SP-A2. Moreover, the ability of SP-A2 to bind with higher affinity to a wider variety of sugars than SP-A1 indicates that the structural pattern or other attributes of SP-A2 make it more favourable for more diverse carbohydrate binding [205]. Similar observations with regard to oligomerization patterns have been made with *in vivo*–expressed SP-A1 and SP-A2 variants in humanized transgenic mice [206]. These *in vivo* experiments also showed that both SP-A1 and SP-A2 gene products are necessary for the formation of the extracellular structural form of surfactant, the tubular myelin. In summary, the available data indicate that *in vitro*– or *in vivo*–expressed single gene products are functional, although with some qualitative and structural differences between the two gene products [195].

3.2.2.4 Regulation of SP-A expression

SP-A is undetectable in human foetal lung tissue during the early part of the second trimester (prior to the differentiation of the alveolar epithelium) [207]. Differentiated type II cells that contain lamellar bodies are observed in human foetal lung by ~22 weeks of gestation, and active surfactant secretion into amniotic fluid occurs after ~30 weeks of gestation. Immunoreactive SP-A can be detected in amniotic fluid at 30 to 32 weeks of gestation [208].

SP-A gene expression is regulated by several growth factors, hormones, and regulatory agents [185]. SP-A mRNA and protein levels are increased by treatment with cAMP analogs [209], epidermal growth factor [210], IFN-γ [211], prostaglandins [212], oxygen [213, 214], endotoxins [215, 216], and β-adrenergic agonists [217]. SP-A protein lelves are decreased by insulin [218] and transforming growth factor (TGF-β) [210]. Also, TNF-α [219], and 12-O-tetradecanoyl-phorbol-13-acetate [220] decreased SP-A mRNA and protein levels. Both inhibitory and stimulatory effects of glucocorticoids on SP-A expression have been reported, with the inhibitory effect observed at higher hormone concentrations [221, 222].

Abnormalities in SP-A levels have been detected in several diseases. For example, SP-A levels are decreased in the amniotic fluid of diabetic pregnant mothers [223, 224]. Pregnancies characterized by low levels of SP-A protein in the amniotic fluid are associated with an increased risk of the infant being born with respiratory distress syndrome [225].

SP-A protein levels are increased in BAL of patients with pulmonary alveolar proteinosis, sarcoidosis, and hypersensitivity pneumonitis [226-228]. Additionally, an increase of SP-A protein levels in BAL of asthmatic patients has been observed, however it is unclear whether this SP-A is functional or it is modified by factors secreted by eosinophils, since SP-A from asthmatics patients has been found to have diminished its anti-inflammatory activity [229]. BAL SP-A levels in patients with both cystic fibrosis and lower airway infections are higher than in cystic fibrosis patients without infection [230]. This increase may occur in response to infection. Additionally, BAL and serum SP-A levels in patients with AIDS-related pneumonia are increased when compared to normal healthy subjects [231]. This increase is not pathogen specific and is seen in infections with *P. carinii* and non-*P. carinii*. Whether the increase in SP-A in AIDS-related pneumonia is a cause or an effect of infection is unclear. Serum SP-A levels have been also reported in patients diagnosed with idiopathic pulmonary fibrosis [229] and combined pulmonary fibrosis and emphysema [232], probably due to extensive damage of lung tissue, leading to transfer of alveolar contents to the blood.

However, a decrease in BAL SP-A protein levels was observed in patients with bacterial and viral pneumonia [233, 234]. Decreased BAL SP-A levels have also been reported in bronchopulmonary dysplasia with infection in baboons [235], in interstitial pneumonia with collagen vascular disease [236], and in smokers versus non-smokers

[229]. Decreased SP-A levels are observed in lavage of patients with idiopathic pulmonary fibrosis [229, 236, 237].

3.2.2.5 SP-A beyond the alveolus

The major site of SP-A synthesis is the lung; however it has been also detected in extra-pulmonary mucosal tissues [238]. SP-A mRNA and/or protein have been detected in the conducting airways [197, 239, 240], middle ear and paranasal sinuses [241, 242], gastrointestinal tract [243, 244], reproductive tract (vagina and prostate) [245], spleen [246], thymus [245], mesothelium, synovium (pleura, pericardium, and peritoneum) [247], and ocular system [248, 249]. The presence of SP-A in these extrapulmonary sites, especially in mucosal tissues, which are largely exposed to pathogens and external particles, supports the important role of SP-A in host defence.

3.2.3 - Immunoprotective role of SP-A

SP-A is one of the soluble factors that contribute to host defence and to create an anti-inflammatory state in the lungs through various mechanisms. SP-A recognizes pathogen-associated molecular patterns on some microorganisms, resulting in aggregation, opsonisation, or permeabilization of microorganisms and facilitation of microbial clearance [11, 250]. It has been reported that SP-A-deficient mice show decreased microbe clearance from the alveolar space and increased tissue markers of inflammation [251]. One of the mechanisms through which SP-A protects the lung of respiratory pathogens is through synergy with other components of the alveolar fluid, such as the antimicrobial peptide SP-B^N, which leads to improved clearance of *Klebsiella pneumonia* [250]. Moreover, SP-A is also able to bind to membrane receptors present in macrophages, epithelial cells, and lymphocytes, modifying their response to different stimuli [11]. These findings make SP-A's protective role in alveolar immune defence evident.

3.2.3.1 Anti-infective roles of SP-A

There is evidence that SP -A limits infection through its ability to bind pathogens. This binding in some cases exerts a direct microbicidal effect and in other

cases promotes pathogen agglutination facilitating its removal by phagocytosis [11] (Fig. 8).

- **Direct Microbicidal Activity**

SP-A performs this activity mainly because its capability to bind to various ligands existing on the surface of microbes, such as rough LPS on Gram-negative bacteria, glycoprotein on fungi, lipoarabinomannan on mycobacteria, phospholipids on mycoplasma, and glycoproteins on virus surface [11].

It has been shown that SP-A directly inhibits the growth of some Gram-negative bacteria such as *E. coli*, *Bordetella pertussis*, *Legionella pneumophila* and *P. aeruginosa* by increasing bacterial membrane permeability [252-255]. It has been suggested that bacterial permeabilization and killing require interaction between SP-A and LPS, most likely through the protein binding to the proximal core and/or lipid A moieties of LPS [256]. However, SP-A has no direct bactericidal effect against Gram-negative respiratory pathogens such as *Klebsiella pneumonia*; but SP-A synergizes with the antimicrobial peptide SP-B^N found in the alveolar fluid to bind, kill and enhance phagocytosis of *K. pneumonia* [14]. This effect of SP-A acting in synergy with other humoral defence alveolar factors is another mechanism that may explain the greater susceptibility of SP-$A^{-/-}$ mice to respiratory pathogen infections [11].

There are no reports about direct killing of SP-A in Gram-positive bacteria however; it has been shown that SP-A is able to inhibit the growth of other bacterial groups, as mycoplasma or mycobacteria [257, 258].

Direct activity of SP-A on virus can be referred to as viral neutralization. It has been shown that SP-A binds to influenza A viruses (via its sialic acid residues) and inhibits recognition of host cell and entry of the viral genome into the target cell [259]. Additionally, SP-A can interact with herpes simplex virus type 1 [260], respiratory syncytial virus [261] and human immunodeficiency virus neutralizing their infectivity [262].

It has been also reported that SP-A has a direct killing effect on some species of fungi. SP-A has been reported to inhibit the growth of *Histoplasma capsulatum* by altering the cell membrane [263]. The interaction of SP-A with fungi seems to be directed by two mechanisms: binding to structural polysaccharides consisting of repetitions of the same oligosaccharide elements, and interaction with glycosylated proteins expressed by fungi on their surface [170].

- **Agglutination and Enhancement of Phagocytosis**

SP-A is able to interact through its C-terminal domain with a variety of molecules present in bacterial, viral or fungal surfaces (i.e. LPS, and other glycoproteins and glycolipids). This feature allows SP-A to agglutinate molecules or microorganisms enhancing mucociliary removal of inhaled dangerous particles, preventing the attachment of pathogens to cell surfaces, and/or inhibiting microbial colonization, invasion and dissemination [170].

SP-A is capable to opsonize microorganisms facilitating uptake by phagocytic cells without the necessity of SP-A interaction with the cell [264, 265]. However, the interaction of SP-A with specific membrane receptors located in alveolar immune cells, such as scavenger receptor A or Myo18A, results in the enhancement of pathogen phagocytosis [266]. Furthermore, SP-A is able to indirectly stimulate phagocytosis by up-regulating the expression of immune cell-surface receptors that are involved in microbial recognition, like the macrophages cell receptors scavenger receptor A [267], complement receptor 3 [268] or mannose receptor [269].

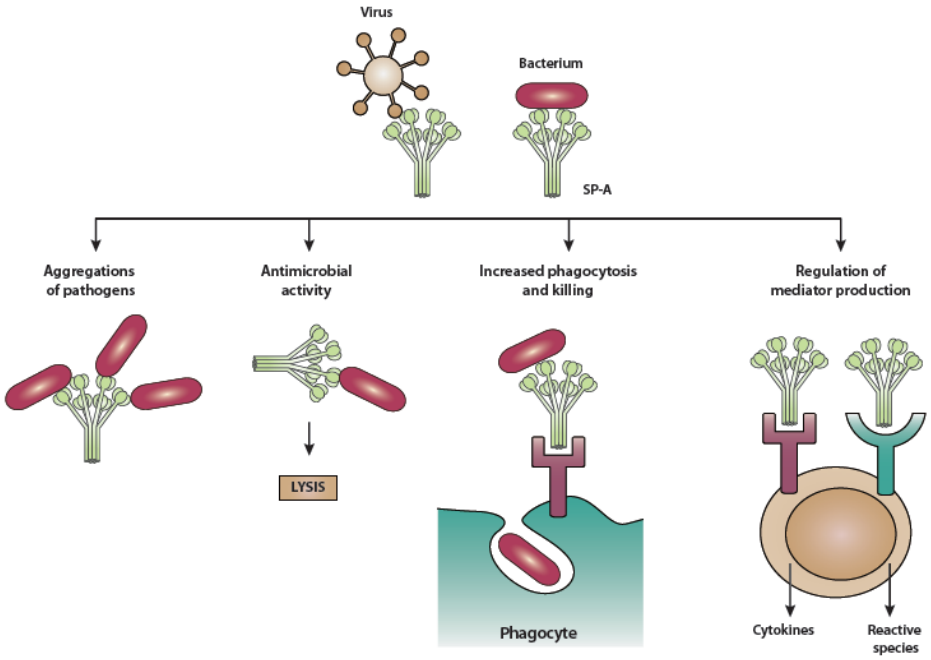


Figure 8. SP-A functions in lung innate defence against infection. SP-A is able to agglutinate and increase phagocytosis of microbes, as well as to act in synergy with the antimicrobial peptide SP-B^N to bind, kill and increase phagocytosis of pathogens such as *K. pneumoniae* . In addition, SP-A modulates phagocytosis and production of inflammatory mediators through interaction with several immune cells receptors. Modified from [11].

3.2.3.2 Immuno-regulatory functions of SP-A and SP-A's receptors

Another level on which SP-A participates in the immune regulation relies on its capacity to modulate leucocyte function by binding to a vast repertoire of receptors present in these immune cells (Fig. 9 and table 2) [11]. Studies involving SP-A null mice showed that these mice exhibited an augmented inflammatory response to a variety of stimuli, suggesting an anti-inflammatory role of SP-A [256].

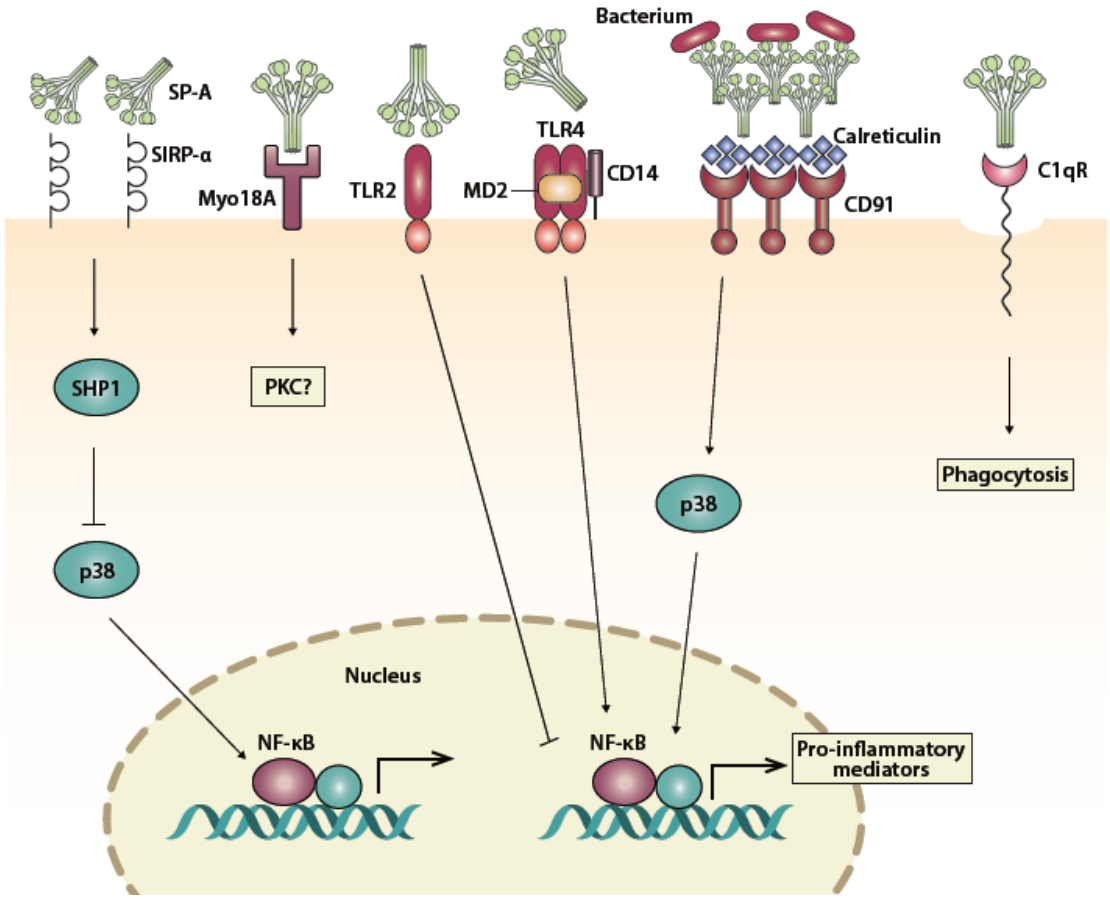


Figure 9. Surfactant protein A (SP-A) receptors. SP-A potentially binds several receptors, including Myo18A, Toll-like receptor 2 (TLR2), TLR4, signal-inhibitory regulatory protein-α (SIRP-α) and CD91-calreticulin. SP-A binds to SIRP-α and inhibits production of inflammatory mediators. By contrast, when SP-A is bound to a pathogen or cellular debris, its collagen-like region is bound to CD91-calreticulin and induces inflammatory-mediator production. Taken from [11].

Table 2. Summary of SP-A receptors on immune cells. Taken from [270]

Receptor	SP-A domain	Expressed by	Functions
Myo18A	Collagen-like tail	T-cells, epithelial type II cells and macrophages	Inhibits T-cell proliferation, enhances bacterial phagocytosis and exerts immuno-regulatory functions on macrophages
TLR2	---	Macrophages	Modulates inflammatory responses against zymosan or peptidoglycan
TLR4 and MD-2	CRD region	Macrophages	Attenuates cell surface binding of smooth LPS and subsequent NF-kB activation
CD14	Neck region	Macrophages	Inhibits smoothLPS/CD14 interactions
Calreticulin-CD91 complex	Collagen-like tail	Macrophages	Enhances phagocytic uptake of apoptotic cells
C1qRp (CD93)	---	Myeloid lineage of hematopoietic cells	Removes pathogens and immune complexes via phagocytosis
SIRPα	Globular domain	Macrophages	Inhibits pro-inflammatory mediators
CD11b (CR3)	Asparagine-linked glycosilation moieties and collagen-like domain	Alveolar macrophages	Augments phagocytosis

- **Effect of SP-A on macrophages**

Numerous *in vitro* and *in vivo* studies in alveolar macrophages have shown that SP-A plays an important anti-inflammatory role by suppressing TLR-ligands activity on these cells [256, 271-275]. SP-A blocks the binding of TLR ligands to their receptors by direct SP-A interaction with TLR4 [273], TLR2 [276], the TLR co-receptor MD2 [276], and CD14 [16, 17]. Additionally, SP-A modifies signalling cascades elicited by TLR ligands in macrophages. For example, SP-A inhibits LPS-induced stimulation of macrophages through increasing the expression of negative regulators of TLR-signalling, such as IRAK-M [277] and β-arrestin 2 [278]. SP-A has been shown to reduce the production of reactive oxygen intermediates by inhibiting NADPH oxidase activity in human monocyte-derived macrophages activated by PMA or serum-opsonized zymosan [279]. Some effects of SP-A inhibiting TLR-ligands-induced signalling have been proved to require SP-A endocytosis. For example, SP-A-induced PKCζ activation and IκBα stabilization, which results in diminished NFκB activation upon LPS stimulation, requires SP-A endocytosis by macrophages [280]. Moreover, SP-A inhibition of IκBα, ERK, p38, and Akt phosphorylation in macrophages stimulated with TLR2 and TLR4 ligands, required SP-A internalization [281].

With regard to the effect of SP-A on IFN-γ-induced classical activation of macrophages, it has been shown that SP-A modulates alveolar macrophages-immune response to IFN-γ however; contradictory results have been reported. One study suggested that SP-A enhances production of NO and iNOS in rat alveolar macrophages stimulated with IFN-γ or INF-γ plus LPS [282]. In contrast, other studies indicate that SP-A suppresses NO production by IFN-γ and IFN-γ plus *M. avium*-stimulated murine alveolar macrophages [283] or IFN-γ plus *M. tuberculosis*-activated murine alveolar macrophages [284]. Similarly to these contrasting results about SP-A effect on IFN-γ-stimulation of macrophages, few studies suggested an enhancing effect of SP-A on LPS-treated THP-1 and Raw264.7 cell lines [285-288]. Although co-purified proteins obtained with different methods of SP-A isolation have been suggested to be responsible of either attenuating or enhancing response of macrophages to LPS [288], the three contrasting studies about the effect of SP-A on IFN-γ-stimulation of macrophages have been done with SP-A extracted by the butanol/octylglucoside method. Therefore, different modulation of LPS/IFN-γ-induced classical activation of macrophages by SP-A may depend on other factors rather than on the SP-A isolation method used.

Although a host of reports indicate a suppressive role of SP-A during macrophage activation, a few studies have shown that SP-A has a role in promoting inflammation [266, 289, 290]. Furthermore, Gardai and collaborators [42] showed that, in a model of peritoneal inflammation, SP-A, promotes phagocytosis as well as inflammation by binding to pathogen-associated molecular patterns or apoptotic cells through its globular heads and to calreticulin-CD91 on macrophages with its collagenous tails. Alternatively, interaction of the signal-regulatory protein SIRPα (which mediates a so-called 'do not eat me' signal) on macrophages with the globular heads of SP-A suppresses macrophages inflammatory responses and phagocytosis.

Another receptor of SP-A is Myosin 18A (Myo18A or MyoXVIIIA), also called surfactant protein A receptor 210 (SP-R210) [291]. This SP-A receptor has been shown to mediate SP-A enhanced phagocytosis of the bacillus Calmette-Guérin and *Staphylococcus aureus* by macrophages [266, 292]. The two isoforms of Myo18A (the large, Myo18Aα, and the short isoform, Myo18Aβ) have been found to regulate expression and/or internalization of other receptors on macrophages, such as scavenger receptor A (SR-A), CD14 or CD36, and the short isoform has been found to physically associate with SR-A and CD14 to enhance response to LPS, suggesting additional mechanisms through which SP-A may modulate macrophages [288]. These contrasting results indicate that, depending on the environmental cues; SP-A can have pro- or anti-inflammatory effect on macrophages, which may be determined by the interaction of the tail or the head of SP-A with different receptors on the alveolar macrophages [42].

The effect of SP-A on the alternative activation of macrophages is less well defined. It has been suggested that SP-A may contribute to the alternative activation of macrophages as it has been shown that SP-A induces the expression of CD206, which is a commonly used marker of alternative activation [13, 269]. However, until now no studies have been made about the effect of SP-A on IL-4/IL-13 stimulation of alveolar macrophages.

- **SP-A targets other immune cells**

Besides the effects of SP-A on alveolar macrophages there is evidence that SP-A targets other cells of the immune system. *In vitro* and *in vivo* studies have indicated that SP-A attracts neutrophils [293, 294]. It has been suggested that SP-A performs neutrophil chemotactic activity by stimulating the release of neutrophil chemotactic

factors by alveolar type II pneumocytes [295]. However, SP-A represses the production and secretion of IL-8 by isolated eosinophils stimulated with ionomycin [296]. Additionally, SP-A expression is up-regulated in patients with allergic rhinitis as compared to controls and positively correlated with eosinophil numbers in the basement membrane of the epithelium, suggesting a key role in mediating inflammation associated with this disease [297].

It has been shown that SP-A interacts with antigen-presenting cells (APCs) and T cells, thereby linking the innate and adaptive immune systems [297] (Fig. 10). It has been shown that SP-A inhibited bone-marrow derived dendritic cells maturation and their phagocytic and chemotactic function [11]. SP-A has been reported to inhibit T-cell proliferation via two mechanisms: an IL-2-dependent mechanism observed with T cells that have been stimulated with plant lectins, CD3-specific antibodies or phorbol esters and an IL-2-independent mechanism that potentially involves downregulation of calcium signalling [11]. However, it has been shown that SP-A enhances T cell proliferation at low strength of signal imparted by exogenous mitogens, specific antibodies, APCs or in homeostatic proliferation, whereas SP-A inhibits T lymphocytes proliferation at higher strength of signal [298]. Additionally, SP-A has been shown to favour T regulatory cells differentiation in a TGF-β-dependent manner [299].

These effects of SP-A on adaptive immune cells have been proposed to be one of the mechanisms of the protective role of SP-A in allergic airway diseases. It has been demonstrated that SP-A reduce allergen-induced lymphocyte proliferation and histamine release in peripheral blood mononuclear cells harvested from children with asthma [300]. *In vivo* studies have shown that SP-A null mice are more susceptible to allergic bronchopulmonary aspergillosis, an allergic disorder induced by *Aspergillus fumigatus*, and exogenous administration of SP-A reduced *A. fumigatus* associated, T cell-mediated, inflammatory indices [301]. SP-A null mice also have enhanced susceptibility to allergic airway disease induced by the single antigen model ovalbumin (OVA). It has been shown that SP-A null mice treated with OVA have greater eosinophilia, T_H2-associated cytokine levels, and IgE levels as well as elevated proportions of $CD4^+$ effector memory T cells in their lungs compared to wild-type mice treated with OVA [302].

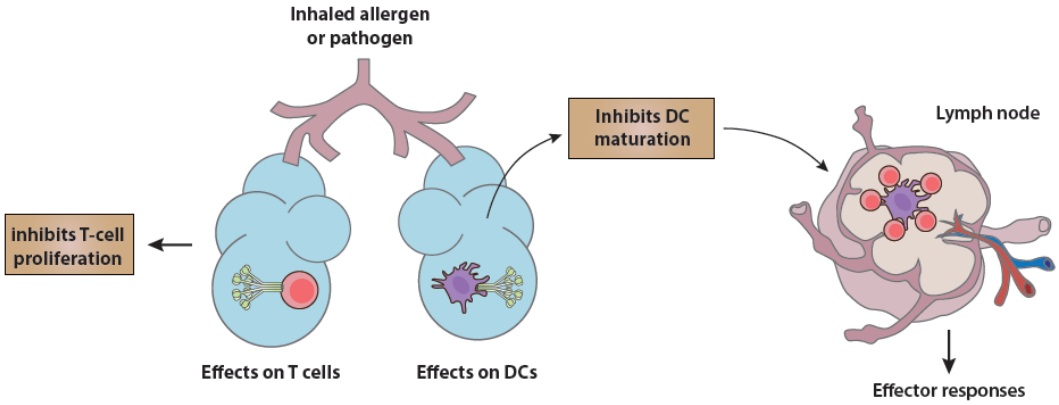


Figure 10. Surfactant protein A (SP-A) regulates dendritic-cell and T-cell functions, thereby providing a link between innate and adaptive immunity. SP-A has been shown to inhibit the maturation of dendritic cells (DCs). Additionally, SP-A inhibits T-cell proliferation. As DCs migrate to the lymph nodes and undergo a maturation process, they acquire the ability to present antigen and activate T cells in the lymph nodes. Modified from [11].

V. OBJECTIVES

Lung respiration moves massive amounts of air containing hundreds of microorganisms and particles over the respiratory surface. Most, but not all, inhaled contaminants are trapped on the mucus layer coating the nasal epithelium and upper respiratory tract. Organisms and particles that reach the alveolar compartment are deposited on the alveolar lining fluid, a thin aqueous film containing lung surfactant [303] that participates in alveolar innate immune defense, especially due to the activity of surfactant proteins SP-A and SP-D [11].

The major effector cells of innate immunity in the alveolar fluid are resident alveolar macrophages (aMϕs) that constitute a unique class of professional phagocytes [3, 4]. Some proteins and peptides present in the alveolar fluid, such as SP-A, constitute the humoral arm of innate immunity in the alveoli. Macrophages recognize danger signals through receptors capable of inducing specialized activation programs. The so-called classical activation of macrophages is induced by TLR-ligands and/or IFN-γ, which trigger a harsh pro-inflammatory response that is required to kill pathogens [3, 4]. Macrophages also acquire alternative activation by IL-4 and IL-13, which trigger a different phenotype that is important for the immune response to parasites and tissue repair [3, 4]. Recent studies have demonstrated that a hallmark feature of macrophage activation by IL-4 is the induction of macrophage proliferation, which may be necessary to generate sufficient numbers of macrophages for pathogen control or wound repair [5, 102, 103, 304].

It is known that SP-A inhibits macrophage activation induced by TLR-ligands and some microorganisms [11, 303]. Many studies have demonstrated that SP-A inhibits macrophage activation induced by LPS [16, 17], zymosan [305], and peptidoglycan [276] by binding to different components of TLR4 and TLR2 receptors. In contrast, the action of SP-A on IFN-γ-activated macrophages is less clear [282-284], and the effect of SP-A on the alternative activation of macrophages remains unknown. As most evidence suggests an anti-inflammatory effect of SP-A on resident alveolar macrophages [11, 303], we hypothesized that SP-A may regulate alveolar macrophage activation, probably reducing the pro-inflammatory response induced by IFN-γ or IFN-γ plus LPS and promoting the alternative phenotype induced by IL-4.

The **main objective** of this thesis was to **evaluate the effect of SP-A on classical activation, induced by IFN-γ or IFN-γ plus LPS, and on alternative activation of alveolar macrophages induced by IL-4.**

This thesis comprises three chapters with the following **specific objectives**:

1. **To evaluate the effect of SP-A on classical activation of alveolar macrophages induced by IFN-γ in the presence or absence of LPS.**

Rationale: *SP-A has been reported to modulate alveolar macrophage immune responses to IFN-γ in the absence or presence of LPS or pathogens; however, there are contradictory reports: While it was shown that SP-A enhances production of NO and iNOS in rat alveolar macrophages stimulated with IFN-γ or INFγ plus LPS [282], other studies indicate that SP-A suppresses NO production by IFN-γ– and IFN-γ plus pathogen–stimulated murine alveolar macrophages [283]. Besides, the molecular mechanism by which SP-A could modulate alveolar macrophage responses to IFN-γ is unknown. To understand the modulatory effects of SP-A on the response of aMϕs to IFN-γ and IFN-γ+LPS stimuli, we studied the effect of SP-A on IFN-γ-stimulated human and rat alveolar macrophages and human M-CSF-derived macrophages (Mϕ(M-CSF)) in the absence or presence of LPS. The results of this chapter show that SP-A has an anti-inflammatory effect on rat and human aMϕs and human Mϕ(M-CSF), counteracting the stimulation exerted by IFN-γ and/or LPS, and* disclose *a novel mechanism by which SP-A controls IFN-γ-induced inflammation in the alveolus.*

2. To discover the role of SP-A during IL-4Rα-mediated alternative activation and proliferation of alveolar macrophages and to investigate whether SP-A might be a tissue-specific factor that determines the ability of macrophages to respond to IL-4.

Rationale: *It has been shown that SP-A enhances the expression of CD206 [13, 269], a widely used marker of Mϕ alternative activation. This observation led us to hypothesize that SP-A could favour IL-4-induced alternative activation of alveolar macrophages. To address this question and to determine the mechanism by which SP-A could modulate alternative activation of macrophages, we treated mouse, rat, and human alveolar macrophages with IL-4 in the presence and absence of SP-A. In*

addition, we compared the response of alveolar macrophages from wild type vs. SP-A-deficient mice to exogenous IL-4 treatment in vivo. We also determined which receptor mediates SP-A effects on IL-4-stimulated alveolar macrophages, in vitro and in vivo. Finally, the physiological relevance of SP-A's effect on the alternative activation and proliferation of alveolar macrophages was assessed in a model of Nippostrongylus brasiliensis infection. The larvae of this parasite migrate transiently through the lung, causing tissue disruption and promoting alternative activation of lung macrophages, which mediate tissue repair and parasite control [306].

To determine whether SP-A acts directly and specifically on alveolar macrophages, adherence-purified macrophages from the alveolar and peritoneal spaces were treated with IL-4, and the ability of SP-A to enhance Mϕ proliferation or activation was compared with that of C1q, which was chosen as a control because it is a protein structurally homologous to SP-A in its supra-trimeric assembly and collagen tail. The response of macrophages from wild type vs. C1qa-deficient mice to exogenous IL-4 treatment was studied. Finally, to ascertain the physiological relevance of C1q-dependent enhancement of type 2 responses, we evaluated the role of C1q in a murine model of peritoneal fibrosis. The results of this chapter show that SP-A and C1q act in a tissue-specific manner to enhance IL-4Rα-mediated macrophage proliferation and activation with consequences for tissue repair, parasite killing, and fibrosis. Critically, both the ligands (SP-A in the lung, C1q in the peritoneal cavity) and their Myo18A receptor are highly conserved across mammals, and we demonstrated that the interaction also takes place in human macrophages.

3. **To study the mechanisms by which SP-A enhances IL-4Rα-mediated macrophage activation and proliferation.**

Rationale: *In chapter 2 we identified the unconventional myosin Myo18A as the SP-A receptor involved in the enhancement of IL-4Rα-mediated macrophage activation and proliferation. Blockade or silencing of Myo18A completely abrogated the effects of SP-A on IL-4-treated alveolar macrophages, suggesting that the synergy between SP-A and IL-4 must occur downstream from their respective receptors. To explore this hypothesis, we treated rat alveolar macrophages with IL-4 and SP-A in the presence of pharmacological inhibitors of IL-4 and SP-A signaling pathways. The results of this chapter show that the pro-proliferative and pro-M2 effects of SP-A require intracellular*

activation of Akt and PKCζ, respectively, which in turn depend on the SP-A-induced upstream activation of PI3K.

VI. CHAPTER 1

Surfactant protein A prevents IFN-γ/IFN-γR interaction and attenuates classical activation of human alveolar macrophages

The research results presented in this chapter have been published in:

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Abstract

Lung surfactant protein A (SP-A) plays an important function in modulating inflammation in the lung. However, the exact role of SP-A and the mechanism by which SP-A affects IFN-γ-induced activation of alveolar macrophages remains unknown. To address these questions, we studied the effect of human SP-A on rat and human alveolar macrophages stimulated with IFN-γ, LPS, and combinations thereof, and measured the induction of pro-inflammatory mediators, as well as SP-A´s ability to bind to IFN-γ or IFN-γR1. We found that SP-A inhibited [IFN-γ+LPS]-induced TNF-α, iNOS, and CXCL10 production by rat alveolar macrophages. When rat macrophages were stimulated with LPS and IFN-γ separately, SP-A inhibited both LPS-induced signaling and IFN-γ-elicited STAT1 phosphorylation. SP-A also decreased TNF-α and CXCL10 secretion by *ex vivo* cultured human alveolar macrophages and M-CSF-derived macrophages stimulated by either LPS, IFN-γ, or both. Hence, SP-A inhibited up-regulation of IFN-γ-inducible genes (*CXCL10, RARRES3,* and *ETV7*) as well as STAT1 phosphorylation in human M-CSF-derived macrophages. In addition, we found that SP-A bound to human IFN-γ (K_D =11 ± 0.5 nM) in a Ca^{2+}-dependent manner and prevented IFN-γ interaction with IFN-γR1 on human alveolar macrophages. We conclude that SP-A inhibition of [IFN-γ+LPS]-stimulation is due to SP-A attenuation of both inflammatory agents and that the binding of SP-A to IFN-γ abrogates IFN-γ effects on human macrophages, suppressing their classical activation and subsequent inflammatory response.

Introduction

Pulmonary surfactant is a lipoprotein complex that lines the alveolar surface. Its main function is to reduce alveolar surface tension [9]; however, it also functions as a modulator of immune responses. The two principal surfactant components involved in innate immunity in the alveolus are surfactant proteins A (SP-A) and D (SP-D) [11].

SP-A is an oligomeric extracellular protein that is found mainly in the alveolar fluid, associated with surfactant extracellular membranes that line the alveolar epithelium and with alveolar cells. SP-A recognizes pathogen-associated molecular patterns on some microorganisms, resulting in aggregation, opsonization, or permeabilization of microorganisms and facilitation of microbial clearance [11, 250]. Moreover, SP-A is also able to bind to membrane receptors present in macrophages, epithelial cells, and lymphocytes, modifying their response to different stimuli [11]. It has been reported that SP-A-deficient mice show decreased microbe clearance from the alveolar space and increased tissue markers of inflammation [251]. These findings make SP-A's protective role in alveolar immune defense evident.

The major effector cells of innate immunity in the alveolus are the alveolar macrophages (aMϕs) that constitute a unique class of professional phagocytes [2]. Macrophages change their phenotype reversibly in response to stimuli. This process is called macrophage activation, which varies from classical activation to alternative activation [73]. The term ‘‘classical activation" refers to macrophages stimulated with IFN-γ (host factor) and pathogen products (e.g., TLR agonists such as LPS) [73]. IFN-γ is the main cytokine associated with classical activation of macrophages. It is mainly produced by T_H1 and natural killer cells and exerts its effects through interactions with its IFN-γ receptor complex, composed of the IFNγR1 and IFNγR2 chains, whose cytoplasmic tails are associated with JAK1 and JAK2 kinases respectively. IFN-γ binds to IFNγR1 with high affinity and activates crosslinking of two molecules of each IFNγR1 and IFNγR2 chain, which results in activation of tyrosine kinases JAK1 and JAK2 and phosphorylation of STAT1. Activated P-STAT1 translocates to the nucleus, where it mediates the transcription of IFN-γ-induced genes such as CXCL10 or IRF transcription factors, among others [74, 307]. On the other hand, the transmembrane TLR4 serves as the primary mediator of LPS signaling, which leads to activation of NF-κB, MAPK, AP-1, IRFs, and early growth response family members, many of which participate in the IFN-γ response [308]. LPS-stimulated macrophages produce pro-inflammatory molecules such as TNF-α, IL-1β, and CXCL10, among others [73, 308]. IFN-γ can ‘prime' macrophages to give an

enhanced response to TLR ligands, such as LPS [92, 93]. Synergy between IFN-γ and LPS occurs at multiple levels, ranging from signal recognition to convergence of signals at the promoters of target genes [92]. Thus the presence of both IFN-γ and TLR ligands induces resting macrophages to rapidly acquire a set of effector functions (production of inflammatory cytokines, chemokines, and reactive oxygen species) that contribute to microbial clearance.

SP-A has been reported to inhibit LPS-induced signaling (p-IκBα, p-ERK, p-p38, and p-Akt) in human monocyte-derived macrophages [281] and to increase expression of the negative regulators of LPS-induced signaling in murine [278] and human monocyte-derived macrophages [277]. SP-A also inhibited the production of pro-inflammatory mediators by human [309] and rat [276, 310] aMϕs, human monocyte-derived macrophages [279], and human macrophage-like U937 cells [16, 17, 273, 276] stimulated with LPS and other TLR ligands. Moreover, SP-A has been reported to modulate aMϕs responses to IFN-γ in the absence or presence of LPS or pathogens, but contradictory observations have also been made. For example, Stamme et al. [282] have shown that SP-A enhances production of NO and iNOS in rat aMϕs stimulated with IFN-γ or IFN-γ plus LPS. In contrast, other studies indicate that SP-A suppresses NO production by murine aMϕs stimulated with IFN-γ– and IFN-γ plus *M. avium* [283] or IFN-γ plus *M. tuberculosis* [284].

To understand the modulatory effects of SP-A on the response of aMϕs to IFN-γ and IFN-γ+LPS stimuli [Mϕ(LPS/IFN-γ)], we studied the effect of SP-A on IFN-γ-stimulated human and rat alveolar macrophages and human M-CSF-derived macrophages (Mϕ(M-CSF)) in the absence or presence of LPS. Our data show that SP-A reduces IFN-γ-triggered inflammation in rat and human aMϕs and human Mϕ(M-CSF). SP-A inhibition of LPS/IFN-γ-induced macrophage stimulation is due to SP-A attenuation of both inflammatory agents. Our findings show, for the first time, that SP-A binds to IFN-γ preventing IFN-γ interaction with IFN-γR1 on the cell surface. We conclude that this could be one of the mechanisms by which SP-A attenuates IFN-γ effects.

Materials and Methods

Isolation, purification and characterization of human SP-A

Surfactant protein A was isolated from bronchoalveolar lavage of patients with alveolar proteinosis using the sequential butanol and octylglucoside extraction [173, 177]. Endotoxin content of isolated human SP-A was about 300 pg endotoxin/mg SP-A as determined by Limulus amebocyte lysate assay (Lonza, Basel, Switzerland). The purity of SP-A was checked by one-dimensional SDS-PAGE in 12 % acrylamide under reducing conditions and mass spectrometry. SP-A consisted of supratrimeric oligomers of at least 18 subunits (MW, 650 KDa). The oligomerization state of SP-A was assessed by electrophoresis under nondenaturing conditions [173, 177], electron microscopy [173], and analytical ultracentrifugation as reported elsewhere [177]. Each subunit had an apparent molecular weight of 36 kDa. Biotinylated SP-A was prepared using the Mini-biotin-XX protein labeling kit (Invitrogen, Carlsbad, CA) as previously described [311]. The structure and functional activity of biotinylated SP-A was similar to that of unlabeled SP-A.

Animal handling and human lung tissue procurement

Rat aMϕs were obtained from Sprague Dawley male rat lungs. Rats (approximately 350 g) were anesthetized with Ketamine (Merial, Duluth, Georgia) (80 mg/kg) and Xylazine (Bayer, Leverkusen, Germany) (10 mg/kg). The cardiopulmonary block was extracted to perform bronchoalveolar lavages with PBS, 0.2 mM EDTA. All animals received humane care in accordance with the Guide for the Care and Use of Laboratory Animals [312] and Spanish guidelines for experimental animals.

As a source of human lung tissue we used multiple organ donors. The review board and the ethics committee of the Hospital Clinic of Barcelona, as well as the Spanish and Catalan Transplant Organizations, approved this study, which was conducted in accordance with the guidelines of the World Medical Association's Declaration of Helsinki. Donors with recent history of tobacco smoking, obesity or any radiological pulmonary infiltrate were excluded from this study. Immediately after obtaining the lungs, we performed a bronchoalveolar lavage at 4°C using 1 liter of 0.9 % NaCl to isolate human aMϕs.

Isolation and culture of primary alveolar macrophages

Bronchoalveolar cells were separated from lavage fluid by centrifugation (250 x g, 7 min). The sedimented cells were washed twice with PBS and the cell pellet resuspended in RPMI 1640 medium (10 % heat-inactivated FBS, 100 U/ml penicillin, 100 µg/ml streptomycin, supplemented with glutamine 2 mM) (Lonza). Human and rat aMϕs were purified by adherence for 90 min at 37°C under a 95% air-5 % CO_2 atmosphere in 150-cm^2 culture flasks as previously reported [309, 313, 314]. Adherent cells were 94.0 ± 1.1 % viable (trypan blue exclusion test). To evaluate the purity of the isolated human macrophages, cells were cytospun in a CytoSpin 3 Cytocentrifuge (Shandon Scientific Ltd, Waltham, Massachusetts) and the cytospin preparations were stained by Diff-Quick kit (Diagnostics Grifols S. A., Barcelona, Spain) following the manufacturer's protocol. Four fields of each sample were counted. Adherent cells were found to be composed of 94.8 ± 0.8 % of aMϕs. On the other hand, flow cytometry analysis of rat macrophages immunostained with anti-CD11c (AbD Serotec, Kidlington, UK) confirmed the purity of rat aMϕ preparations.

Incubation Conditions

Adherent cells were gently scraped, plated in 96-well plastic dishes (7.5 x 10^4 cells per well) in 0.2 ml of RPMI with 5 % FBS and precultured overnight. Cells were incubated for another 24 h in the presence or absence of smooth LPS (*Escherichia coli* 055:B5, 1 ng/ml) (Sigma, St. Louis, Missouri), either rat or human recombinant IFN-γ (Calbiochem, Darmstadt, Germany) (0.05-10 ng/ml), human SP-A (5, 12.5, 25 and 50 µg/ml), and combinations thereof. Higher doses of both LPS (10-100 ng/ml) and IFN-γ (100 ng/ml) were also assayed. At the SP-A concentrations used the effect of SP-A was greater at lower doses of LPS and/or IFN-γ than at higher doses. Cell viability was higher than 97 % under assay conditions. Macrophage cultures were plated in triplicate wells, and each series of experiments was repeated at least three times.

Generation of human M-CSF-derived macrophages

Human PBMC were isolated from buffy coats from normal donors over a lymphoprep gradient (Nycomed Pharma, Oslo, Norway), according to standard procedures. Monocytes were purified from PBMC by magnetic cell sorting using CD14 microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany). Monocytes (95 % $CD14^+$ cells) were cultured at 0.5 x 10^6 cells/ml for 7 days in RPMI 1640 supplemented with 10 % FBS at 37°C in a humidified atmosphere with 5 % CO^2, and containing M-CSF

(ImmunoTools, Friesoythe, Germany) (10 ng/ml) to generate M-CSF-derived macrophages as previously described [315]. Cytokines were added every 2 days. Cells were treated with human recombinant IFN-γ (1 ng/ml), human SP-A (50 μg/ml), and combinations thereof on the 8th day. Cultures were plated in triplicate wells, and each series of experiments was repeated at least three times.

Cytokine determinations

Secreted cytokines were quantified in supernatants of treated human and rat aMϕs using specific ELISA kits following the supplier's instructions. Rat and human TNF-α and human CXCL10 ELISA kits were purchased from BD Biosciences (San Diego, CA) and rat CXCL10 from Peprotech (Rocky Hill, NJ). In brief, antibodies were coated on a 96-well Nunc-Immuno Plate MaxiSorp Surface (Thermo Scientific, Waltham, Massachusetts) in 0.1 M sodium carbonate, pH 9.5, overnight. After blocking with PBS, 10% FBS, and extensive washing, samples and standards were incubated for two hours at room temperature. Cytokines were detected with biotinylated detection antibodies and streptavidin-horseradish peroxidase. The colorimetric reaction was developed with tetramethylbenzidine (BD Biosciences) and was stopped with 4 M sulfuric acid (Sigma), and the absorbance at 450 nm was read on an ELISA reader (DigiScan; Asys HiTech GmbH, Eugendorf, Austria).

Western blot analysis

Cells were lysed with three freezing-thawing cycles in a buffer containing: 10 mM HEPES, pH 7.9, 15 mM MgCl, 10 mM KCl, 0.5 mM EDTA, 0.2 % Triton X-100, 1 mM benzamidine, 200 μg/ml aprotinin, 200 μg/ml leupeptin, and 1 mM phenylmethylsulfonyl fluoride (Sigma). When phosphorylated proteins were analyzed, phosphatase inhibitors were added to the buffer: 20 mM β-glycerophosphate, 10 mM NaF, 10 mM sodium pyrophosphate, and 2 mM orthovanadate (Sigma). Samples were resolved by SDS-PAGE in reducing conditions and transferred to polyvinylidene fluoride membranes (Bio-Rad, Hercules, California). After blocking with 2.5 % skim milk, membranes were washed in PBS, 0.1% tween, and incubated with anti-iNOS, GAPDH, P-ERK, total ERK, P-STAT1, and total STAT1 (Cell Signaling, Danvers, Massachusetts). Membranes were incubated with horseradish-peroxidase-labeled anti-rabbit IgG (Sigma), then washed and exposed to ECL reagents (Merk Millipore, Darmstadt, Germany). Immunoreactive bands were densitometered (Quantity One Software, BioRad) and then

normalized to GAPDH for iNOS quantification and to the respective total protein for quantification of phosphorylated proteins.

Quantitative real-time RT-PCR

Total RNA was extracted using an RNeasy kit from Quiagen (Venlo, Netherlands), retrotranscribed and amplified in triplicates with the reverse transcription system kit (Applied Biosystems, Waltham, Massachusetts) [316]. Oligonucleotides for selected genes were designed according to the Universal Probe Library system (Roche Diagnostics, Rotkreuz, Switzerland) for quantitative real-time PCR (qRT-PCR). The analyses for selected genes were made by lightcycler 480 (Roche). For quantification of selected genes, the next oligonucleotides were used: 5´-aagcagttagcaaggaaaggtc-3´ and 5´-gacatatactccatgtagggaagtga-3´ for *CXCL10*, 5´-gacccaggggtctgttc-3´ and 5´-aaggagcagctgatacacgtaa-3´ for *ETV7*, 5´-ctcctcttggcttcgagatg-3´ and 5´-aaggcggaaaatctcaatca-3´ for *RARRES3*, and 5´-agccacatcgctcagacac-3´ and 5´-gccaatacgaccaaatcc-3´ for *GAPDH*. Levels of mRNA were quantitated using ΔΔCt method [317] and were then normalized to maximal expression levels obtained in the presence of IFN-γ.

Solid-phase binding assays

Solid-phase binding assays were performed as previously described [311] with minor modifications. Either rat IFN-γ, human IFN-γ, human sIFN-γR1 (R&D, Minneapolis, MN), or human serum albumin (HSA) (Sigma) (1 μg per well) was coated on a 96-well maxisorp microtiter plate in 0.1 mM sodium bicarbonate buffer, pH 9.5, overnight at 4°C. The wells were washed three times with buffer A (5 mM Tris-HCl, pH 7.4, containing 150 mM NaCl) with 0.1 mM EDTA. Wells were blocked with buffer A with 0.1 mM EDTA containing 5 % skim milk for 2h. After the plate was washed, biotinylated SP-A, in concentrations ranging from 0 to 470 nM (0 to 333 μg/ml), was added to the wells in buffer A in the presence or absence of 2 mM $CaCl_2$. Incubations were performed for 1 h at RT. After extensive washing, streptavidin-horseradish peroxidase (Sigma) was added to the wells. To assay the inhibition of the IFN-γ/sIFN-γR1 interaction by SP-A the mixture of IFN-γ (0.1 μg/ml) and SP-A (in concentrations ranging from 0 to 160 μg/ml) was added to the sIFN-γR1-coated wells in buffer A, with or without 2 mM $CaCl_2$, and incubated at RT for 1 h. The binding of IFN-γ to sIFN-γR1 was detected using a polyclonal anti-human IFN-γ (Abcam, Cambridge, UK) and horseradish peroxidase-conjugated anti-rabbit antibody. The binding of either biotin-labeled SP-A or anti-human IFN-γ was detected with tetramethylbenzidine.

The colorimetric reaction was stopped with 4 M sulfuric acid, and the absorbance was read at 490 nm on an ELISA reader.

Dynamic light scattering

The hydrodynamic diameters of human and rat IFN-γ, human sIFN-γR1, and human SP-A, as well as mixtures of these components, were determined at 25°C in a Zetasizer Nano S (Malvern Instruments, Malvern, UK) equipped with a 633-nm HeNe laser as previously reported [311]. Six scans were performed for each sample, and all of the samples were analyzed in triplicate. The interaction of SP-A with IFN-γ in solution was measured by the addition of different SP-A concentrations (from 0 to 150 nM; 0-100 μg/ml) to 322 nM (7.7 μg/ml) IFN-γ in buffer A in the absence and presence of 175 μM or 2.5 mM $CaCl_2$.

Binding of [^{125}I]-IFN-γ to IFN-γR1 at the cell surface of human alveolar macrophages and inhibition by SP-A

Recombinant human IFN-γ was labeled with [^{125}I]-Bolton Hunter reagent (PerkinElmer, Waltham, Massachusetts) as described previously [318]. In brief, 10 μg of human IFN-γ in 10 μl of 0.1 M sodium borate buffer, pH 8.5, was added to 0.7 mCi of [^{125}I]-Bolton Hunter reagent, and the reaction mixture was agitated for 15 minutes at RT. The reaction was stopped by the addition of 0.5 ml of 0.2 M glycine in 0.1 M borate buffer, pH 8.5 for 5 minutes at RT. Free-iodine was separated from the labeled protein using disposable PD-10 desalting columns (GE Healthcare, Waukesha, WI) according to manufacturer's instructions. The concentration of the labeled IFN-γ was determined by sandwich-type ELISA by using a polyclonal anti-human IFN-γ, a horseradish peroxidase-conjugated anti-rabbit antibody and tetramethylbenzidine for color detection.

Human aMϕs were plated in 96-well plates (1 x 10^5 cells per well) in 0.2 ml of RPMI containing 5% FBS and primed overnight with LPS (1 ng/ml) (37°C, under 5 % CO_2 atmosphere). Control wells with no cells were coated with the same medium supplemented with 5% HSA. Subsequently, the cells were washed with PBS. Wells were blocked with PBS plus 0.5% HSA for 30 minutes at 4ºC. After the plate was washed with PBS, [^{125}I]-IFN-γ (10 ng/ml) and SP-A (in concentrations ranging from 0 to 100 μg/ml) were added to the cells in RPMI either with 2.5 mM $CaCl_2$ or with 5 mM EDTA. Incubations were performed for 2 h at 4ºC. After extensive washing, the cells were lysed with 10% SDS, and radioactivity was counted with a Perkin Elmer Wallac Wizard 1470-020 Gamma Counter. To confirm that the detected [^{125}I]-IFN-γ bound specifically to its receptor at the

cellular surface, we used a mouse mAb (BD Biosciences) (50 μg/ml), which recognizes the region of human IFN-γR1 that binds to IFN-γ, to inhibit IFN-γ interaction with its receptor [307]. An irrelevant, isotype-matched mouse IgG1 mAb (AbD Serotec) was used as a control for these experiments.

Statistics

Data are presented as means ± SEM. Differences in means between groups were evaluated by one-way ANOVA followed by the Bonferroni multiple-comparison test. An α level ≤ 5% ($P \leq 0.05$) was considered significant.

Results

SP-A inhibits LPS and IFN-γ effects on rat alveolar macrophages

To determine the effects of SP-A on the classical activation of aMϕs, primary aMϕs isolated form rat lungs were activated with LPS and/or IFN-γ in the absence and presence of SP-A. Figures 1A and 1B show that LPS induced significant TNF-α secretion and iNOS expression in rat aMϕs, and that the induction of both factors was even greater when macrophages were simultaneously incubated with LPS plus IFN-γ. This indicates that IFN-γ treatment results in efficient priming/amplification of TLR4-induced cellular responses. However, IFN-γ alone did not induce TNF-α secretion and iNOS expression in rat aMϕs, which is contrary to previous results in RAW 264.7 peritoneal macrophages cell line [319], where IFN-γ up-regulates TNF-α and iNOS production.

When different concentrations of SP-A were tested (5, 12.5, and 25 μg/ml), we found that SP-A significantly inhibited, in a dose-dependent manner, TNF-α secretion (Figure 1A) and iNOS production (Figure 1B) by rat aMϕs stimulated with either LPS alone or LPS plus IFN-γ. The effect of SP-A on LPS-stimulated rat aMϕs is consistent with previous results [16, 17, 276, 309]. In line with previous findings [281], SP-A also significantly inhibited LPS-induced phosphorylation of ERK1/2 (T202/Y204) in a dose-dependent manner (Figure 2A and B), whereas ERK activation was not significantly induced in the presence of either SP-A alone or IFN-γ (Figure 2A and B).

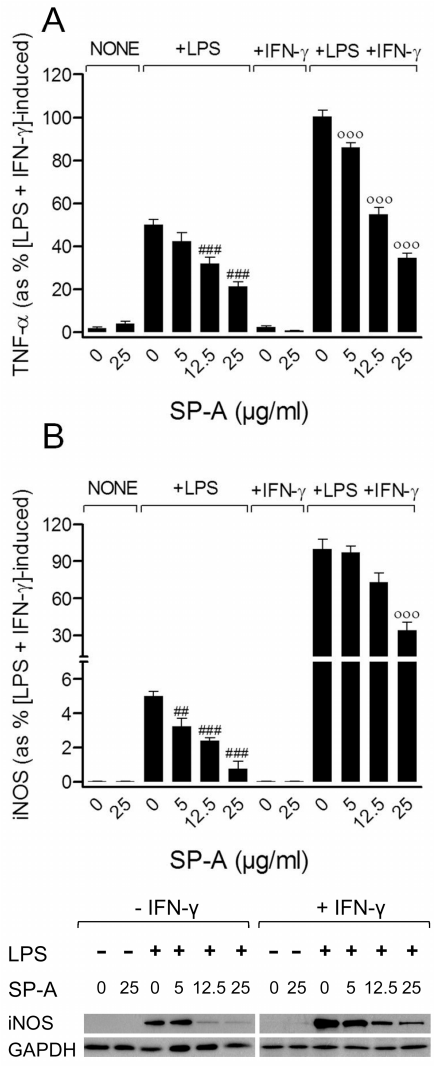


Figure 1. Inhibitory effect of SP-A on TNF-α and iNOS production by rat alveolar macrophages stimulated with IFN-γ and/or LPS. Purified rat aMφs were cultured in the presence or absence of IFN-γ (10 ng/ml), LPS (1 ng/ml), SP-A (5, 12.5, 25 μg/ml), and combinations thereof. We measured TNF-α secretion by ELISA (A) and iNOS production by Western blot (B) after 24 hours of IFN-γ and/or LPS-stimulation. The results are expressed as percent of LPS plus IFN-γ-stimulated TNF-α and iNOS production in the absence of SP-A. The mean value of LPS plus IFN-γ-induced TNF-α secretion in the absence of SP-A was 6.8 ± 0.5 ng/ml (100%). The data shown are means ± SEM of three different aMφs cultures. $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ when compared with the response elicited by LPS in the absence of SP-A. $^{\circ\circ\circ}P < 0.001$ when compared with the response elicited by LPS+ IFN-γ without SP-A.

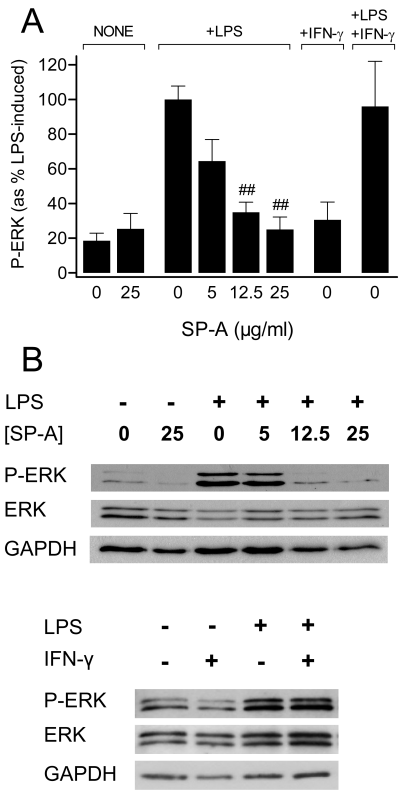


Figure 2. Inhibitory effect of SP-A on LPS induced ERK phosphorylation by rat alveolar macrophages. Purified rat aMϕs were cultured in the presence or absence of LPS (1 ng/ml), IFN-γ (10 ng/ml), SP-A (5, 12.5, 25 μg/ml), and combinations thereof. We measured ERK phosphorylation by Western blot after 30 minutes of LPS-stimulation in the presence or absence of SP-A or IFN-γ. In A, the results are presented as means (± SEM) from three different aMϕs cultures, and expressed as percentages of LPS-induced ERK phosphorylation. $^{\#\#}P < 0.01$ when compared with the response elicited by LPS in the absence of SP-A. In B, representative Western blot images of ERK phosphorylation in rat aMϕs exposed to either LPS and/or SP-A (upper panel) or LPS and/or IFN-γ (lower panel) are shown.

SP-A attenuates IFN-γ effects on rat alveolar macrophages

IFN-γ signaling is mediated by the cytosolic factor STAT1 that is activated during IFN-γ-dependent JAK-STAT activation [92]. STAT1 is phosphorylated at two sites (tyrosine 701 and serine 727) following IFN-γ exposure [92]. To determine whether the inhibitory effect of SP-A on aMϕs stimulated with LPS plus IFN-γ was also due to SP-A attenuation of IFN-γ, we measured the effect of SP-A on IFN-γ-induced STAT1 Y701 phosphorylation and CXCL10 secretion by rat aMϕs (Figure 3).

We found that SP-A dose-dependently inhibited IFN-γ-stimulated CXCL10 secretion (Figure 3A) and STAT1 Y701 phosphorylation (Figure 3B, C) by rat aMϕs. LPS alone had no effect on STAT1 Y701 phosphorylation after 30 minutes of LPS-stimulation (Figure 3B, D). This was expected since S727, but not Y701, is phosphorylated by p38 MAPK activated by TLR agonists [92]. However, LPS alone induced CXCL10 secretion (137 ± 15 pg/ml) by rat aMϕs, and simultaneous stimulation with LPS plus IFN-γ induced higher CXCL10 secretion than with IFN-γ alone (305 ± 12 pg/ml and 172 ± 12 pg/ml, respectively) (Figure 3A). The CXCL10 promoter contains response elements for STAT1, NF-kB, and AP-1 [92], which would explain why aMϕs produce CXCL10 after stimulation with IFN-γ and/or LPS. Secretion of CXCL10 induced by LPS, IFN-γ, or LPS plus IFN-γ

was also inhibited by SP-A in a dose-dependent manner (Figure 3A), consistent with the results obtained with TNF-α and iNOS described above. Together, these data demonstrate that SP-A inhibition of [IFN-γ+LPS]-stimulation is due to SP-A attenuation of both inflammatory agents.

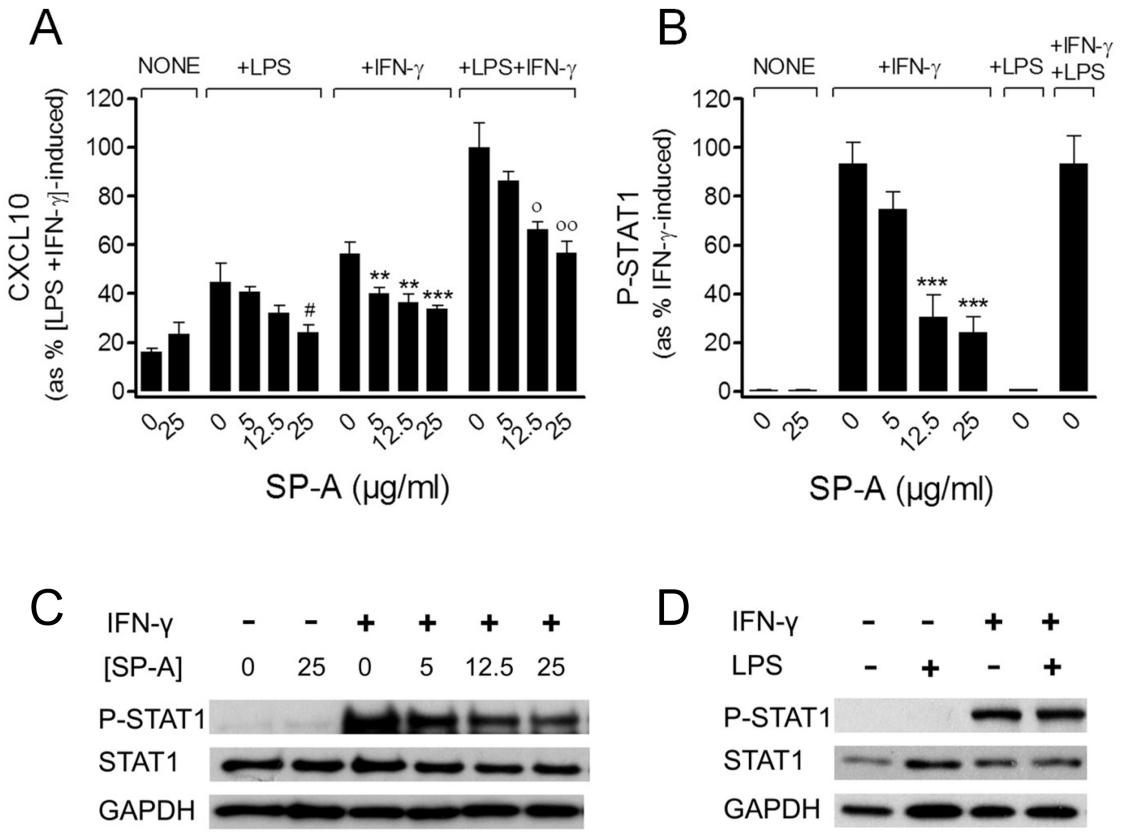


Figure 3. Inhibitory effect of SP-A on IFN-γ-induced STAT1 Y701 phosphorylation and CXCL10 secretion by rat alveolar macrophages. Purified rat aMφs were cultured in the presence or absence of IFN-γ (1-10 ng/ml), LPS (1 ng/ml), SP-A (5, 12.5, 25 μg/ml), and combinations thereof. (A) CXCL10 secretion was measured after 24 hours IFN-γ (1 ng/ml) and/or LPS-stimulation by ELISA. The results are presented as means (± SEM) from three different cell cultures and expressed as percentages of LPS plus IFN-γ-induced CXCL10 secretion. The mean value of LPS plus IFN-γ-induced CXCL10 secretion by rat aMφs in the absence of SP-A was 305 ± 12 pg/ml (100 %). (B) STAT1 Y701 phosphorylation was measured after 30 minutes stimulation with IFN-γ (10 ng/ml), LPS (1 ng/ml), or both, by Western blot. The data shown are means ± SEM of three different aMφs cultures and were expressed as percentages of IFN-γ-induced STAT1 phosphorylation. (C, D) Representative Western blot images of STAT1 phosphorylation in rat aMφs exposed to either IFN-γ (10 ng/ml) and/or SP-A (C) or IFN-γ (10 ng/ml) and/or LPS (1 ng/ml) (D) are shown. $^{\#}P < 0.05$ when compared with LPS stimulation without SP-A. $^{**}P < 0.01$, $^{***}P < 0.001$ when compared with the response elicited by IFN-γ alone in the absence of SP-A. $^{\circ}P < 0.05$, $^{\circ\circ}P < 0.01$ when compared with the response elicited by LPS+IFN-γ without SP-A.

SP-A inhibits LPS and IFN-γ effects on *ex vivo* cultured human alveolar macrophages

Given the above set of data on rat aMϕs, we sought to extend our findings to the case of primary human aMϕs. Figure 4A shows that SP-A inhibited TNF-α secretion by human aMϕs stimulated with LPS alone or LPS plus IFN-γ in a dose-dependent manner. Similarly to the observations using rat aMϕs presented above, IFN-γ itself was not capable of significantly stimulating TNF-α secretion. However, IFN-γ increased the levels of LPS-induced TNF-α secretion by human aMϕs, as previously reported [313], and this further increase was significantly impaired by SP-A. Therefore, similar to the case of rat aMϕs, SP-A is capable of limiting the production of pro-inflammatory cytokines by human aMϕs exposed to the classical activation stimuli LPS+IFN-γ.

Moreover, as shown in Figure 4B, SP-A dose-dependently inhibited IFN-γ-induced CXCL10 secretion by *ex vivo* cultured human aMϕs. Simultaneous stimulation with IFN-γ and LPS induced higher CXCL10 secretion than with IFN-γ alone (367 ± 36 pg/ml and 268 ± 16 pg/ml, respectively), and SP-A also inhibited [LPS+ IFN-γ]-induced CXCL10 secretion (Figure 4B). On the other hand, iNOS protein did not increase in response to LPS or LPS plus IFN-γ in human aMϕs (Extended Figure 4.1). This was expected since little to no iNOS and NO have been detected in human macrophages obtained from normal donors [309, 320], mainly due to epigenetic silencing of NOS2 [321].

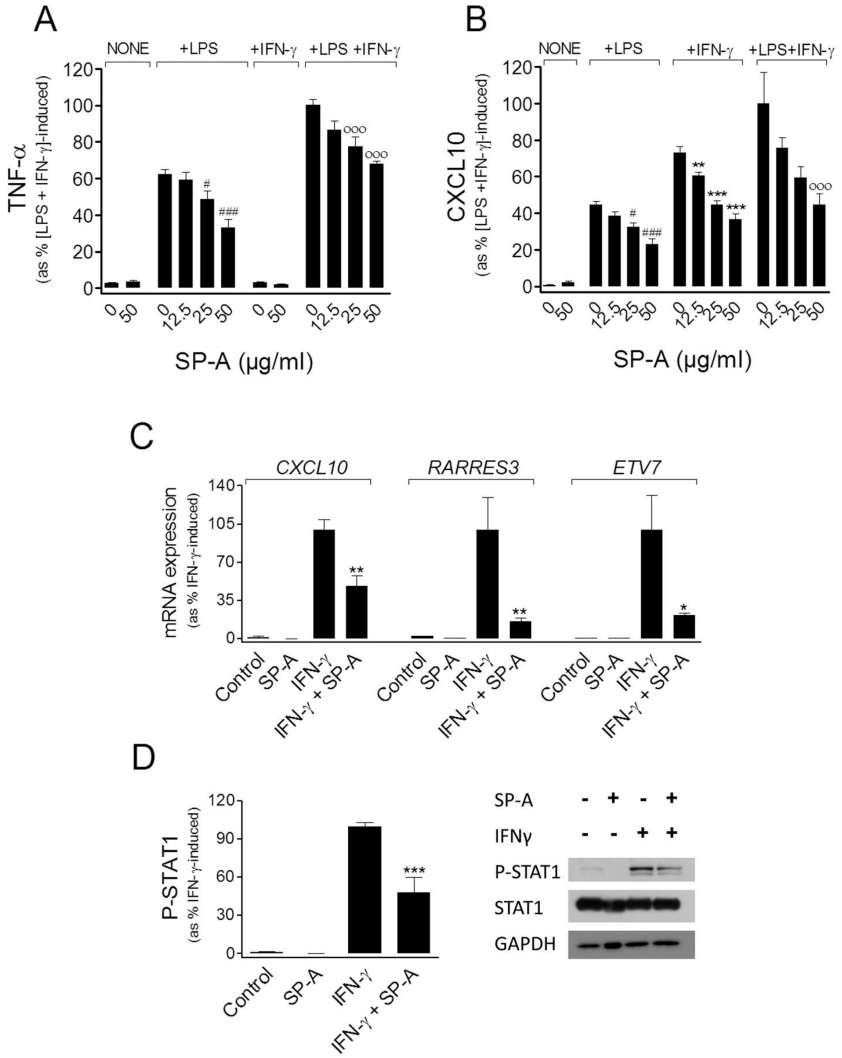
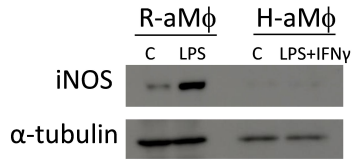


Figure 4. Inhibitory effect of SP-A on IFN-γ and/or LPS-challenged human macrophages. In A and B, human aMϕ were cultured in the presence or absence of IFN-γ (1 ng/ml), LPS (1 ng/ml), SP-A (12.5, 25, 50 μg/ml), and combinations thereof. We measured TNF-α (A) and CXCL10 (B) secretion after 24 h IFN-γ and/or LPS-stimulation. In (C) Human Mϕ(M-CSF) were cultured in the presence or absence of IFN-γ (1 ng/ml), SP-A (50 μg/ml), and combinations thereof. We measured *CXCL10*, *RARRES3,* and *ETV7* induction by RT-qPCR after 24 hours IFN-γ-stimulation. In (D) Human Mϕ(M-CSF) were cultured in the presence or absence of IFN-γ (0.05 ng/ml), SP-A (50 μg/ml), and combinations thereof. STAT1 Y701 phosphorylation was evaluated by Western blot after 30 minutes of stimulation. In (A) and (B) the results are expressed as percent of LPS plus IFN-γ-stimulated secretion of TNF-α (A) or CXCL10 (B) in the absence of SP-A. The mean values of LPS plus IFN-γ-induced TNF-α and CXCL10 secretion in the absence of SP-A were 875 ± 134 pg/ml and 367 ± 36 pg/ml, respectively (100 %). In (C) results were expressed as percentages of IFN-γ-induced gene in the absence of SP-A. In (D) results were expressed as percentages of IFN-γ-induced STAT1 phosphorylation. The data shown are means ± SEM of three different Mϕ cultures. $^{\#}P < 0.05$, $^{\#\#\#}P < 0.001$ when compared with the response elicited by LPS without SP-A. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ when compared with the response elicited by IFN-γ in the absence of SP-A. $^{\circ\circ\circ}P < 0.001$ when compared with the response elicited by LPS+IFN-γ in the absence of SP-A.



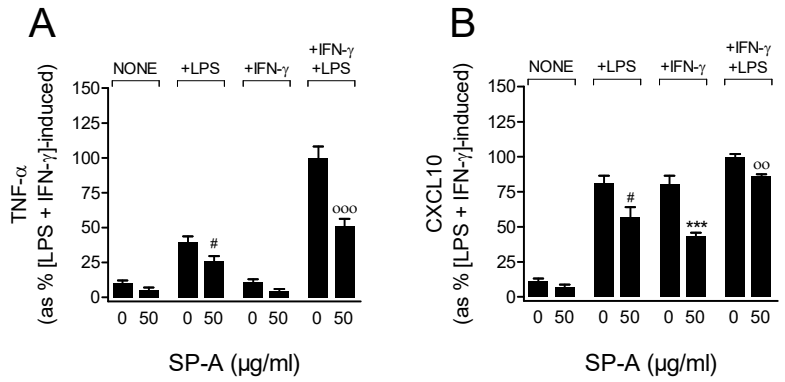
Extended Figure 4.1. iNOS protein is not induced upon LPS+IFN-γ stimulation in human alveolar macrophages. Human and rat aMϕ were cultured in the presence or absence of IFN-γ (10 ng/ml) and/or LPS (10 ng/ml) for 24 hours. iNOS protein expression was examined by Western blot. A representative Western blot image is shown.

SP-A inhibits IFN-γ effects on human monocyte-derived macrophages Mϕ(M-CSF)

We then used human monocyte M-CSF-derived macrophages [human Mϕ(M-CSF)]. Alveolar macrophages show phenotypical features of anti-inflammatory macrophages [1] and M-CSF prime monocytes to differentiate into anti-inflammatory macrophages [322]. As in the case of *ex vivo* cultured human aMϕs, SP-A inhibited TNF-α and CXCL10 secretion by Mϕ(M-CSF) stimulated by LPS, IFN-γ, and LPS+IFN-γ (Extended Figures 4.2 A and B). Human Mϕ(M-CSF) stimulated with IFN-γ and/or LPS led to higher CXCL10 secretion (21.7 ± 1.5 ng/ml, 17.4 ± 2.9 ng/ml, and 13.1 ± 1.7 ng/ml in response to LPS+IFN-γ, IFN-γ, and LPS, respectively) than *ex vivo* cultured human aMϕs (367 ± 36 pg/ml, 268 ± 16 pg/ml, and 164 ± 7 in response to LPS+IFN-γ, IFN-γ, and LPS, respectively).

CXCL10 is one of the paradigmatic genes induced by IFN-γ [92]. To find out whether SP-A affected other IFN-γ-regulated genes, we evaluated the effect of SP-A on the IFN-γ–dependent gene expression in human Mϕ(M-CSF). To that end, human Mϕ(M-CSF) were stimulated with IFN-γ in the presence and absence of SP-A, and IFN-γ-induced genes were analyzed by quantitative real-time RT-PCR. Figure 4C shows that IFN-γ up-regulated the expression of *CXCL10* (fold change 65 ± 6), *RARRES3* (fold change 34 ± 13) identified as a retinoic acid responder gene [323], and *ETV7* (fold change 177 ± 43) (also known as the human ETS family gene TEL2/ETV7, which promotes proliferation and has a role in oncogenesis [324]). Importantly, SP-A inhibited the IFN-γ-induced expression of *CXCL10*, *RARRES3,* and *ETV7* genes by 49 %, 85 %, and 70 %, respectively (Figure 4C). Moreover, we found that SP-A inhibited IFN-γ–stimulated STAT1 Y701 phosphorylation in human Mϕ(M-CSF) (Figure 4D). Together, these findings

demonstrate that SP-A also suppressed IFN-γ effects in the absence and presence of LPS on human Mϕ(M-CSF).



Extended Figure 4.2. SP-A inhibits TNF-α and CXCL10 secretion by human Mϕ(M-CSF) stimulated with IFN-γ, LPS, or IFN-γ +LPS. Human Mϕ(M-CSF) were cultured in the presence or absence of IFN-γ (0.5 ng/ml), LPS (0.5 ng/ml), SP-A (50 μg/ml), and combinations thereof. We measured TNF-α (A) and CXCL10 (B) secretion after 24 h of IFN-γ and/or LPS-stimulation. The results are expressed as percent of LPS plus IFN-γ-stimulated secretion of TNF-a (A) or CXCL10 (B) in the absence of SP-A. The data shown are means ± SEM of four different Mϕ cultures. The mean values of LPS + IFN-γ-induced TNF-α and CXCL10 secretion in the absence of SP-A were 2.6 ± 0.3 ng/ml and 21.7 ± 1.5 ng/ml, respectively (100 %). #P < 0.05, ###P < 0.001 when compared with the response elicited by LPS without SP-A. *P < 0.05, **P < 0.01, ***P < 0.001 when compared with the response elicited by IFN-γ in the absence of SP-A. ooo P < 0.001 when compared with the response elicited by LPS+IFN-γ in the absence of SP-A.

SP-A binds to IFN-γ and prevents IFN-γ binding to its receptor on human alveolar macrophages

To determine the mechanism by which SP-A diminishes IFN-γ effects on rat and human aMϕs as well as human Mϕ(M-CSF), we first studied the potential interaction between SP-A and IFN-γ in a solid phase binding assay. Figure 5A shows that biotinylated SP-A bound to human IFN-γ-coated wells in a dose- and Ca^{2+}-dependent manner, with K_D =11 ± 0.5 nM. Biotinylated SP-A bound neither to IFN-γ-coated wells in the absence of Ca^{2+}, nor to wells coated with human serum albumin, nor to wells containing buffer alone, regardless of the presence of Ca^{2+}. Biotinylated SP-A also bound to rat IFN-γ in a dose- and Ca^{2+}-dependent manner with K_D = 28 ± 4 nM (data not shown). Figure 5B shows that the percentage of biotinylated SP-A binding to human IFN-γ-coated wells decreased by addition of unmodified SP-A, indicating that binding of biotinylated SP-A to IFN-γ-coated wells was not due to the biotin moiety.

In addition, the interaction of SP-A with human and rat IFN-γ was examined in solution by dynamic light scattering. Figure 5C (left panel) shows that human IFN-γ alone displays a major peak, which corresponds to particles with a hydrodynamic diameter of 10.5 ± 0.5 nm. The hydrodynamic diameter of human IFN-γ determined in these experiments approaches the predicted diameter of 7 ± 1 nm, which does not take into account water molecules enclosing the protein in solution [325]. In the case of human SP-A, two identifiable peaks were recognized for SP-A alone, one corresponding to SP-A particles with a hydrodynamic diameter of 38 ± 5 nm and another minor peak corresponding to SP-A aggregates with a hydrodynamic diameter of 1000 nm (data not shown). Self-aggregation of SP-A occurs in a Ca^{2+}- and NaCl-dependent manner [326]. To reduce SP-A self-aggregation, experiments were performed in the presence of 175 μM $CaCl_2$.

Figure 5C (central panel) shows that the addition of increasing concentrations of SP-A (ranging from 0 to 120 nM; 0-80 μg/ml) to an IFN-γ solution (322 nM) containing calcium caused an SP-A concentration-dependent decrease of the IFN-γ peak. At an IFN-γ/SP-A molar ratio of approximately 3:1, only one peak (58 nm) was observed with a hydrodynamic diameter higher than those of SP-A and IFN-γ alone. This new peak likely consists of SP-A/IFN-γ complexes. This peak did not appear in the absence of Ca^{2+} (Figure 5C, right panel). Similar results were obtained with rat IFN-γ (data not shown). Therefore, this set of experiments demonstrates that SP-A and IFN-γ interact in solution in a Ca^{2+}-dependent manner.

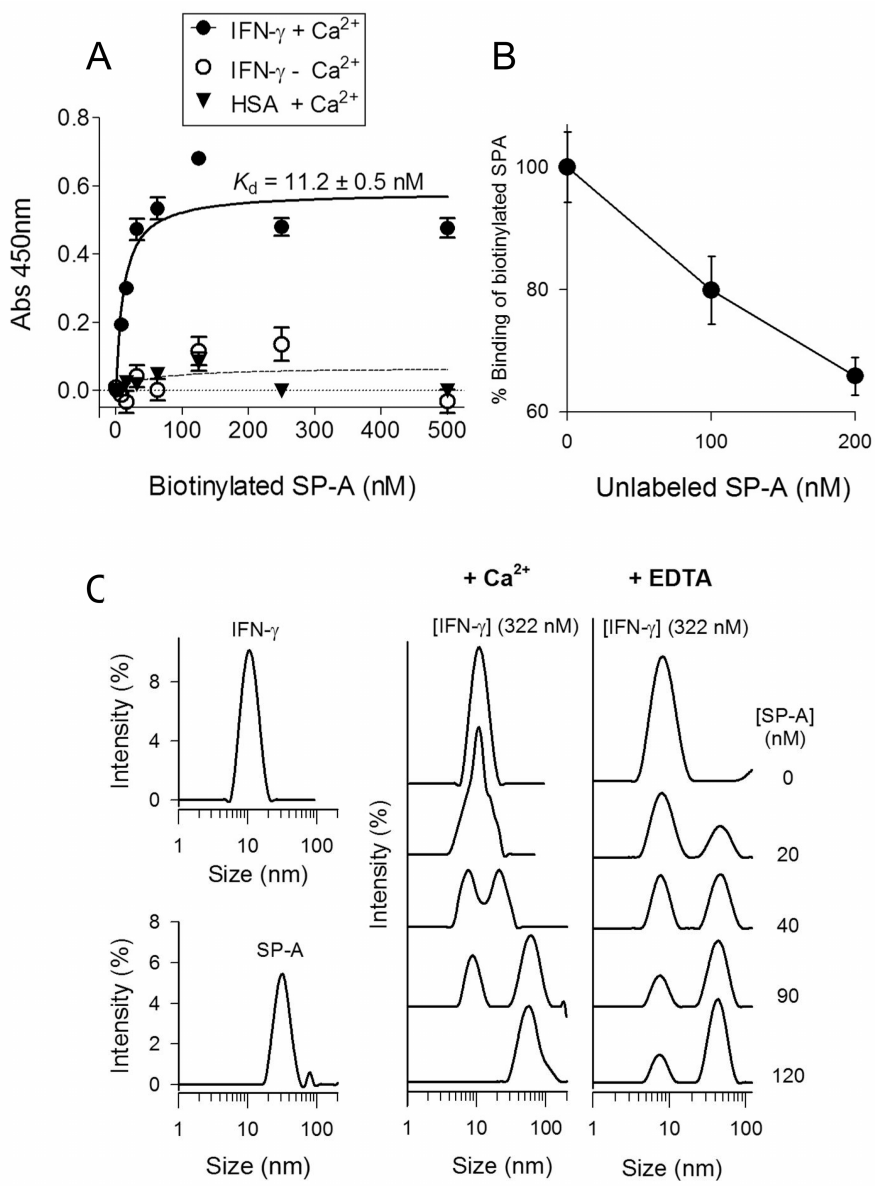


Figure 5. SP-A binds to IFN-γ. (**A**) Either human IFN-γ or human serum albumin (HSA) (1 μg) was coated onto microtiter plate wells. Biotinylated SP-A (0-470 nM; 0-333 μg/ml) was then added to the wells in the presence or absence of 2.5 mM Ca^{2+}, and levels of bound SP-A were determined with streptavidin-horseradish peroxidase. (**B**) **Competition by unlabeled SP-A of biotinylated SP-A binding to** human IFN-γ**.** Unlabeled SP-A was added simultaneously with biotinylated SP-A (100 nM; 80 μg/ml) **to human** IFN-γ-coated wells (1 μg) in presence of 2.5 mM Ca^{2}. In A and B, the data shown are means ± SEM of three different experiments. The assays from each SP-A concentration were performed with six independent determinations. (**C**) DLS analysis of the hydrodynamic diameter of human IFN-γ (10.5 ± 0.5 nm) and SP-A (38 ± 5 nm). The *y* axis represents the relative intensity of the scattered light; the *x* axis denotes the hydrodynamic diameter of the particles present in the solution. The analysis of the hydrodynamic diameter of particles after the addition of increasing concentrations of SP-A (0-120 nM) (0-80 μg/ml) to a solution containing a constant concentration of IFN-γ (322 nM) (7.7 μg/ml) is shown in the presence or absence of calcium. In C, one representative experiment of three is shown.

To clarify whether SP-A has a direct effect on IFN-γ-induced cell response by interacting not only with IFN-γ but also with its cellular receptor, the extracellular domain of IFN-γR1 (sIFN-γR1) was coated onto microtiter plate wells. Biotinylated SP-A was then added to the wells, and the level of bound SP-A was determined with streptavidin-horseradish peroxidase. No detectable binding of SP-A to sIFN-γR1 was observed (Figure 6A). In addition, no detectable SP-A/sIFN-γR1 interaction was observed in solution when examined by dynamic light scattering (Figure 6B).

To determine whether SP-A interferes with the binding of IFN-γ to its receptor, IFN-γ binding to coated sIFN-γR1 was determined by ELISA using a polyclonal anti-human IFN-γ and in the presence and absence of SP-A (Figure 6C). We found that SP-A interfered with the binding of IFN-γ to IFN-γR1 in the presence but not the absence of Ca^{2+}. The inhibition caused by SP-A was saturable and dose- and Ca^{2+}-dependent, with a ~80 % inhibition at concentrations equal to or higher than 20 μg/ml (30 nM). As expected, the binding of IFN-γ to its receptor IFN-γR1 was not affected by Ca^{2+} (data not shown).

We next examined whether SP-A could effectively inhibit the binding of IFN-γ to IFN-γR1 on the cell surface. To achieve this, we analyzed the binding of [^{125}I]-IFN-γ to IFN-γR1 on human aMϕs. To confirm that the detected [^{125}I]-IFN-γ bound specifically to its receptor at the cellular surface, we used a monoclonal antibody (mouse anti-human CD119) that recognizes the region of IFN-γR1 that binds to IFN-γ to block [^{125}I]-IFN-γ binding to its receptor. Figure 6D shows that, in the absence of SP-A, [^{125}I]-IFN-γ bound to human aMϕs and that this binding was specifically abrogated by the blocking antibody. Conversely, an isotype-matched mouse IgG1 control did not inhibit [^{125}I]-IFN-γ binding to human aMϕs (data not shown). However, addition of SP-A clearly inhibited the [^{125}I]-IFN-γ binding to IFN-γR1 in a dose-dependent manner in the presence but not absence of calcium. Similar results were found on rat aMϕs. Therefore, our results indicate that SP-A impairs IFN-γ recognition by IFN-γR1 on the cell surface. This could be at least one of the mechanisms by which SP-A suppresses IFN-γ effects.

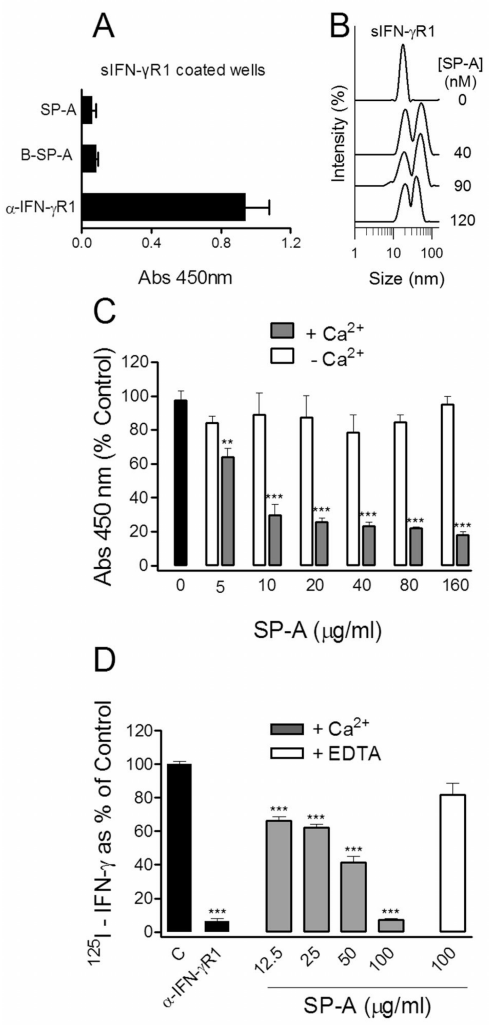


Figure 6. SP-A prevents IFN-γ interaction with IFN-γ receptor 1 (IFN-γR1/CD119). (A) sIFN-γR1 (1 μg) was coated onto microtiter wells. Coated sIFN-γR1 was detected using rabbit polyclonal anti-human IFN-γR1. Either biotinylated (B-SP-A) or unlabeled SP-A (160 μg/ml) was incubated for 1 h in the presence of 2.5 mM Ca^{2+}. SP-A binding to coated sIFN-γR1 was determined with streptavidin-horseradish peroxidase. **(B)** DLS analysis of the hydrodynamic diameter of particles after the addition of increasing concentrations of SP-A (0-80 μg/ml) to a solution containing a constant concentration of sIFN-γR1 (5 μg/ml) (**C**) sIFN-γR1 (1 μg) was coated onto microtiter wells. IFN-γ (100 ng/ml) and different concentrations of SP-A (0-160 μg/ml) in the presence or absence of 2.5 mM Ca^{2+} were incubated for 1 h. The binding of IFN-γ to sIFN-γR1-coated wells was detected using a polyclonal anti-human IFN-γ and horseradish peroxidase-conjugated anti-rabbit antibody. $^{**}P < 0.01$, $^{***}P < 0.001$, when compared with IFN-γ binding without SP-A (defined as 100 %.) (**D**) LPS primed human aMϕs were blocked and incubated with [^{125}I]-IFN-γ (10 ng/ml) and SP-A (at concentrations ranging from 0 to 100 μg/ml) with either 2.5 mM $CaCl_2$ or 2.5 mM EDTA. Incubations were performed for 2 h at 4 ºC. Cells were lysed with 10 % SDS and radioactivity was measured. Human aMϕs incubated with anti-IFN-γR1 antibody were used as a positive control to prevent interaction between IFN-γ and its receptor. The gamma counts of IFN-γ binding without SP-A were defined as 100 %. The data are means ± SEM of three different experiments (with different human aMϕ cultures in D). The assays from each macrophage preparation were performed with triplicate determinations. $^{**}P < 0.01$, $^{***}P < 0.001$, when compared with IFN-γ binding to its receptor in the absence of SP-A.

Discussion

Innate immune defense in the alveolar space is characterized by a delicate balance between an effective inflammatory response and the maintenance of tissue integrity. Alveolar macrophages play a major role in this equilibrium by producing and releasing a variety of biologically active products in response to stimuli such as LPS and IFN-γ [1, 2]. Macrophage activation and the initiation of inflammation involve a complex balancing act between activating and repressing signals. The results of the present study show that SP-A has an anti-inflammatory effect on rat and human aMϕs and human Mϕ(M-CSF), counteracting the stimulation exerted by IFN-γ or LPS+IFN-γ.

In this study we have shown that SP-A, used at concentrations within the ranges found in healthy individuals (15-105 μg/mL) [327], inhibited the production of pro-inflammatory molecules such as TNF-α and CXCL10 by rat and human aMϕs and human Mϕ(M-CSF) stimulated with IFN-γ, LPS, and LPS+IFN-γ. SP-A also inhibited iNOS production by rat aMϕs stimulated with LPS and LPS+IFN-γ. iNOS production by stimulated human aMϕ was not detected, confirming previous results that indicate that human aMϕ and blood-monocyte-derived macrophages obtained from normal donors and stimulated *in vitro* generally do not express iNOS [309, 320, 321]. SP-A also inhibited IFN-γ-induced STAT1 phosphorylation in rat aMϕs and human Mϕ(M-CSF) and inhibited up-regulation of IFN-γ-inducible genes (*CLCX10*, *RARRES3,* and *ETV7*) by human Mϕ(M-CSF). In addition, we have shown for the first time that human SP-A bound to IFN-γ with high affinity (K_D = 11 ± 0.5 nM for human IFN-γ and K_D = 28 ± 4nM for rat IFN-γ) and prevented IFN-γ interaction with its receptor IFN-γR1 on human aMϕs. These data disclose a novel mechanism by which SP-A controls inflammation in the alveolus.

Our observation that SP-A reduces IFN-γ-triggered inflammation in rat and human aMϕs and human Mϕ(M-CSF) is in agreement with the previous studies that showed that SP-A suppresses NO production by murine alveolar Mϕs stimulated with IFN-γ– and IFN-γ plus M. avium [283] or IFN-γ plus M. tuberculosis [284]. However, our results appear to contradict the data from Stamme and colleagues [282] using rat aMϕs. We do not currently have a clear explanation for these contrasting results, although there might be some differences in the experimental design. For instance, starting cell number, media and supplements, different types of plastic, and other conditions such as whether macrophages are rested prior to activation might have substantial effects on activation status. In addition, the source and concentration of cytokines is also important.

Alveolar macrophages reside in a tissue compartment that is constantly exposed to contaminated air. Thus alveolar macrophage activation is tightly controlled through several cell-cell and soluble mediator interactions to limit unwanted inflammatory responses [1]. SP-A is one of the soluble factors that contribute to create an anti-inflammatory state in the lungs through various mechanisms. First, SP-A blocks the binding of TLR ligands to their receptors by direct SP-A interaction with TLR4 [273], TLR2 [276], the TLR co-receptor MD2 [276], and CD14 [16, 17]. Second, SP-A modifies macrophage response to TLR ligands by modulating signaling cascades. For example, SP-A increases the expression of negative regulators of TLR-signaling, such as IRAK-M [277] and β-arrestin 2 [278], thereby inhibiting LPS-induced stimulation of macrophages. Moreover, SP-A promotes PKCζ activation and IκBα stabilization through mechanisms that require SP-A endocytosis by macrophages [280]. Internalized SP-A also inhibits IκBα, ERK, p38, and Akt phosphorylation by macrophages stimulated with TLR2 and TLR-4 ligands [281]. Third, SP-A reduces the production of reactive oxygen intermediates by inhibiting NADPH oxidase activity in human monocyte-derived macrophages activated by PMA or serum-opsonized zymosan [279]. Here we showed that the binding of SP-A to IFN-γ, which suppressed IFN-γ interaction with its receptor IFN-γR at the cell surface, is another mechanism by which SP-A limits inflammation and maintains a tolerant lung environment in the steady state. However, following an infection, a harsh IFN-γ induction would potentially override SP-A capability to block IFN-γ, leading to a desirable inflammatory response to fight against infection. The fact that SP-A is induced in response to IFN-γ [211] suggests that SP-A may also be implicated in the regulation of detrimental inflammation at the resolution phase after infection.

IFN-γ is essential for anti-mycobacterial immunity, and disorders of IFN-γ production confer predisposition to mycobacterial disease in humans [328]. However, high levels of secreted IFN-γ may be harmful. It has been shown that IFN-γ and IFN-γ-induced CXCL10 are directly involved in the exacerbation of different lung inflammatory diseases (acute lung injury and bronchiolitis) in murine experimental models and/or humans [329, 330]. Moreover, IFN-γ causes emphysema and alterations in pulmonary protease/antiprotease balance when expressed in pulmonary tissues [331]. Furthermore, the administration of neutralizing antibodies against IFN-γ or CXCL10 has been shown to attenuate lung injury and/or improve mice survival rate [332, 333].

The alveolar fluid from normal lungs contains high concentrations of SP-A that probably minimize the biological effects of low concentrations of endotoxins that enter the

alveolus and IFN-γ. In patients with acute lung injury in which proinflammatory cytokines and neutrophils accumulate in the air spaces, the concentration of SP-A significantly decreases [334, 335]. The observation that SP-A restores lung tissue integrity in response to sterile inflammation [336] supports the hypothesis that SP-A may be important in modulating inflammation and epithelial integrity in the lung in response to acute injury.

In summary, we have shown that human SP-A inhibits IFN-γ, LPS, and LPS+IFN-γ effects on rat and human aMϕs and human Mϕ(M-CSF) and that SP-A binds to IFN-γ with high affinity, inhibiting IFN-γ recognition by its receptor on the cell surface. These data unravel a previously unknown mechanism by which SP-A/IFN-γ interaction plays a significant role in tipping the balance of inflammation to protect the alveolar epithelium.

VII. CHAPTER 2

Defense collagens, SP-A and C1q, enhance IL-4Rα-mediated macrophage activation and proliferation promoting local repair

Abstract

Macrophages activated by the IL-4Rα are central effectors of type 2 immunity and play important roles in helminth infection, asthma, allergy, and fibrosis. However, tissue-specific enhancers of IL-4Rα-mediated type 2 responses remain unknown. In this study, we found that secreted soluble defense collagens acted as local enhancers of IL-4Rα-mediated macrophage effector functions. In the lung, surfactant protein A (SP-A) enhanced IL-4-dependent proliferation and activation of alveolar macrophages, resulting in better parasite clearance and lung injury resolution following infection with the lung-migrating nematode *Nippostrongylus brasiliensis.* Beyond the lung, the structurally related molecule, C1q amplified type 2 activation of peritoneal macrophages, leading to enhanced dialysis-induced peritoneal fibrosis. SP-A and C1q both generated their effects on macrophages via the unconventional myosin18A (*aka* SP-R210) that acts as a cell surface receptor. Thus, SP-A and C1q are tissue-specific factors that act through myosin18A to amplify local type 2 responses with consequences for lung repair, parasite control, and peritoneal fibrosis.

Introduction

The type 2 cytokines IL-4 and IL-13 trigger a specialized macrophage phenotype (M(IL-4)) that promotes control of helminth infection [337] and localized wound healing to rapidly repair damaged tissue [6, 128, 131]. IL-4 and IL-13 also induce macrophage proliferation, which may be necessary to generate sufficient numbers of macrophages for pathogen control or wound repair [5]. In addition to these beneficial roles, M(IL-4)s contribute to pathology associated with allergy, asthma, and tissue fibrosis [131]. Despite the importance of IL-4Rα signaling throughout the body, little is known about tissue-specific factors that control IL-4/IL-13 mediated effects, with the potential to promote both beneficial and detrimental actions of M(IL-4).

Infection with *Nippostrongylus brasiliensis* is a widely used mouse model for the study of helminth-induced type 2 responses in the lung. Migration of the larvae through the lung causes significant damage to the epithelium and vasculature and alveolar M(IL-4) are essential to mediate lung repair [6]. Alveolar macrophages (aMϕs) together with the respiratory epithelium are covered by pulmonary surfactant, a lipid-protein network that contains associated proteins essential for keeping the alveolus open and for host defense [9, 11]. One of these proteins, surfactant protein A (SP-A), constitutes the major surfactant protein component by weight in the alveolar fluid [11]. SP-A is a versatile recognition protein that binds to a great variety of ligands [11] and is a member of a group of secreted soluble defense collagens. All defense collagens contain a recognition domain contiguous with a collagen-like triple helical domain and play important roles in protecting the host from pathogen entry [338]. Defense collagens oligomerize as trimers or multiples thereof due to their collagen domains. Members of this group include the first component of the complement system (C1q), collectins (e.g., SP-A, SP-D, mannan-binding lectin), ficolins, and adiponectin [338].

The majority of studies on SP-A have focused on bacterial clearance [11, 14] and its role in the context of type 1 immune responses [11, 336, 339]. Here we chose to examine the role of SP-A in the context of helminth-induced type 2 immune responses. We found that SP-A enhanced IL-4-dependent activation and proliferation of aMϕs and, critically, this contributed to resolution of lung injury following *N brasiliensis* infection. We also identified the unconventional myosin, Myo18A (*aka* SP-R210) as the receptor for

SP-A involved in the enhancement of M(IL-4). The identification of Myo18A, which is not restricted to the lung, led us to discover that beyond the alveolus other secreted soluble collagens acted through Myo18A to promote type 2 immunity. Specifically, we show that C1q enhances M(IL-4) proliferation and activation in the peritoneal cavity, promoting dialysis-induced peritoneal fibrosis. Thus, we found that SP-A and C1q are local amplifiers of type 2 responses suggesting that other secreted soluble defense collagens present in distinct tissues might have the same action.

Methods

Proteins. Surfactant protein A was isolated from BAL of patients with alveolar proteinosis using a sequential butanol and octylglucoside extraction [14, 173, 177, 339, 340]. The purity of SP-A was checked by one-dimensional SDS-PAGE in 12 % acrylamide under reducing conditions and mass spectrometry. The oligomerization state of SP-A was assessed by electrophoresis under non-denaturing conditions [173, 177], electron microscopy [173], and analytical ultracentrifugation as reported elsewhere [177]. SP-A consisted of supratrimeric oligomers of at least 18 subunits. Each subunit had a relative molecular mass (Mr) 36 kDa. Recombinant human SP-A1 (SP-A1^{hyp}) was expressed in insect cells and purified from the medium by mannose affinity chromatography [177, 340]. The stability of SP-A1^{hyp} collagen domain was assessed by circular dichroism [177, 340] and differential scanning calorimetry [177]. The oligomerization state of SP-A was evaluated by electrophoresis under non-denaturing conditions [177, 340] and analytical ultracentrifugation [177]. SP-A1^{hyp} consists of trimers and hexamers. The endotoxin content of native or recombinant human SP-A was < 0.1 endotoxin units /mg of SP-A as determined by Limulus amebocyte lysate assay (GenScript, Piscataway, New Jersey). The microbicidal activity of native and recombinant human SP-A against Escherichia coli J5 was evaluated by bacterial killing assays as described previously [14]. Levels of mouse SP-A from BAL, pleural exudate, or lung tissue were detected by Western-blot analysis as reported elsewhere [14, 173, 177, 339, 340]. Native human C1q was obtained from Abcam (Cambridge, UK). C1q consists of octadecameres of 18 subunits with a Mr 410 kDa.

Experimental animals

C57BL6 mice used in this study (SP-A (*Sftpa1*$^{-/-}$) [341], C1q (*C1qa*$^{-/-}$) [342] (kindly provided by Dr. Mohini Gray), and IL-4Rα (*Il4ra*$^{-/-}$) [343] deficient mice and WT mice) were bred and maintained at the University of Edinburgh in specific-pathogen free conditions. Sex-matched mice were 6-8-weeks old at the start of the experiment, and all mice were housed in individually ventilated cages. *Sftpa1*$^{-/-}$ and *Sftpa1*$^{+/+}$ littermates were genotyped using specific primers (**Table 1**) and used for experiments of *N. brasiliensis* infections. Mice were not randomized in cages, but each cage was randomly assigned to a treatment group. Investigators were not blinded to mouse identity during necropsy; however, the analysis of adult worms, eggs in faeces, lung and peritoneal pathology were performed in a blinded fashion. Experiments were performed in accordance with

the United Kingdom Animals (Scientific Procedures) Act of 1986 and the Spanish guidelines for experimental animals. All researchers were accredited for animal handling and experimentation by the UK and Spanish government Home Office. Dispensation to carry out animal research at The University of Edinburgh was approved by the University of Edinburgh Animal Welfare and Ethical Review Body and granted by the UK government Home Office; as such all research was carried under the project licenses PPL70/8548 and PPL70/8470. Sample size was calculated on the basis of the number of animals needed for detection of macrophage proliferation in WT mice, based on published experiments [5, 102]. Data was not excluded under any circumstances.

Table 1. Genotyping of the *Sftpa1*$^{-/-}$ and *Sftpa1*$^{+/+}$ littermates

SP-A-deficient mice genotyping	
Primer	Sequence
Sftpa1 F neo	GTGGGGTGGGATTAGATAAATGC
Sftpa1 1743-1766	GCATTAGACGACAGAACTCCAGCC
Sftpa1 R 1981-1957	TACTGAGAGATGTGTGCTTGGTGAG

Sprague Dawley rats (~350 g) were purchased from Harlan (Indianapolis, IN). All animal experiments were fully compliant with the regulations set by the local ethical committee. Animals were treated according to the Directive 2010/63/EU of The European Parliament and the Spanish act RD53/2013 of 8th February 2013 on protection of animals used for experimentation and other scientific purposes.

***Nippostrongylus brasiliensis* infection**

N. brasiliensis was maintained by serial passage through C57BL/6 mice, as described previously [344]. Mice were infected subcutaneously with 250 or 400 *N. brasiliensis* third-stage larvae. Analysis of samples was performed at day 6 post-infection. Egg output was analysed in faeces and adult worm burden was determined by removing the small intestine and exposing the lumen by dissection. For macrophage proliferation analysis, mice were injected with 100 µl of 10 mg/ml BrdU in Dulbecco's phosphate buffered saline 3h before experimental end-point. The lungs and pleural cavities were washed to obtain the bronchoalveolar lavage (BAL) and pleural exudate. Subsequently, the right lung was perfused and fixed for histology. Alternatively, one section of the left lung was stored for mRNA quantification; another section was

homogenized to obtain single cell suspensions for flow cytometry analysis, and a third section was stored for SP-A quantification. In this case, lung tissue was homogenized in HBSS containing protease inhibitor cocktail. SP-A was detected in both lung homogenates and BAL by Western blot analysis using an anti-mouse SP-A (GeneTex, Inc, Irvine, CA). SP-A levels were normalized by BAL volume or GAPDH. Only samples on which the same BAL volume was recovered were used for SP-A quantification.

BAL cells were obtained by washing the lung with Dulbecco's phosphate buffered saline containing 0.5% BSA (m/v). Cells from pleural or peritoneal exudate were obtained by washing either pleural or peritoneal cavity with RPMI 1640 containing 2 mM L-glutamine, 200 U/ml penicillin, 100 μg/ml streptomycin. Single cell suspensions of thoracic lymph node tissue were re-stimulated ex vivo with *N. brasiliensis* excretory secretory antigen [345] (1 µg/ml) or anti-CD3 (1 µg/ml), and cell supernatants were analysed by ELISA 72 h later.

Peritoneal fibrosis model

Peritoneal fibrosis was induced by continuous administration of Dianeal PD-4 [(Baxter, Deerfield, Illinois), as described previously [346] with slight modifications. Mice received a total of 14 injections of 500 µl (~200 ml/kg) of Dianeal PD-4 (i.p.) on alternate days. Animals were sacrificed a day after the last delivery. The right section of the parietal layer of the peritoneum was collected for histology analysis. The left section of the peritoneum was stored in RNAlater for subsequent analysis. Peritoneal macrophages were isolated from the peritoneal cavity as described below.

Histology

The right lung lobes were perfused and fixed with 10 % neutral buffered formalin, incubated overnight and transferred to 70 % ethanol. Lungs were paraffin-embedded, sectioned and stained with H&E. Linear means intercept (Lmi) method was used to quantify emphysema like damage [347]. To calculate Lmi, 20 random non-overlapping fields (magnification x200) per H&E stained lung sample were taken. Six horizontal lines were drawn across each image (ImageJ 1.44) and the total number of alveolar wall intercepts counted per line. The length of each line was then divided by the number of intercepts to give the Lmi value. Images that included large bronchi and vessels were avoided and analysis was performed in a blinded and randomised fashion. With respect to histological analysis of the peritoneum tissue, the right parietal layer of the

peritoneum was fixed with 10% neutral buffered formalin, incubated overnight and transferred to 70 % ethanol. Subsequently, peritonea were paraffin-embedded, sectioned and stained for Masson's trichrome. The thickness of the submesothelial compact zone was measured using ImageJ and this value was used to score the extent of peritoneal fibrosis. Five independent (×200) images were taken to examine the overall section. For each image, six horizontal lines were randomly drawn across the submesothelial compact zone to measure its thickness and the average value of each sample was used for analysis.

IL-4 complex delivery and *in vivo* blocking of Myo18A

IL-4 was delivered as a 2:1 molar ratio of recombinant mouse IL-4 (Peprotech) and anti-IL-4 mAb (clone 11B11; BioXcell,) [5, 102]. For pulmonary macrophage analysis, mice were injected i.p. with IL-4 complex (IL-4c) containing 5 μg of IL-4 and 25 μg of 11B11, or PBS vehicle control on days 0 and 2. Simultaneously, 50 μg of anti-Myo18A antibody [348] or Rabbit IgG (R&D Systems) were delivered intra-nasally on days 2 and 3, and samples were collected on day 4. For studies of peritoneal macrophages, mice received a single i.p. injection of 1 μg of IL-4 and 5 μg of 11B11, or PBS vehicle control. Anti-Myo18A neutralizing antibody or Rabbit IgG (100 μg) was delivered 2 hours before IL-4c injection and samples were collected 24 hours later. Mice received a pulse of BrdU 3 h before experimental end-point. Bronchoalveolar, pleural and peritoneal cavity lavages as well as lung tissue collection were performed as described above.

RNA extraction and quantitative real-time PCR

A section of the left lung or parietal peritoneum was stored in RNAlater (Ambion, Carlsbad, CA). Tissue was homogenised in Trizol (Invitrogen) with a TissueLyser (Qiagen, Hilden, Germany). Similarly, human alveolar macrophages were collected with Trizol, and RNA was prepared according to manufacturer's instructions. Reverse transcription was performed using 2 µg of total RNA, 50 U Tetro reverse transcriptase (Bioline, London, UK), 40 mM dNTPs (Promega, Fitchburg, WI), 0.5 µg Oligo dT15 (Roche, Basel, Switzerland), and RNasin inhibitor (Promega). Transcript levels of genes of interest were measured by real-time PCR with the Lightcycler 480 II system (Roche) using SYBR Green I Master kit and specific primers **(Table 2)** as previously described [347]. PCR amplification was analysed using 2nd derivative maximum algorithm (LightCycler 480 Sw 1.5, Roche) and the expression of the gene of interest was

normalised to a housekeeping gene *Rn18s*, *Rpl13a,* or *GAPDH*. In the case of human alveolar macrophages, cells from at least 8 humans were used for all groups.

Table 2. Primer sequences used for quantitative RT-PCR of genes of interest.

Gene	Primer	Sequence
Sftpa1	For	CTGGAGAACATGGAGACAAGG
	Rev	AAGCTCCTCATCCAGGTAAGC
Col3a1	For	AAGGGTGAAGTCGGTGCTC
	Rev	TCCAGCTCCACCTCTAGCA
Col1a1	For	TCTGGTCTCCAGGGTCCTC
	Rev	GTCTTTGCCAGGAGAACCAG
Acta2	For	CCAACCGGGAGAAAATGAC
	Rev	CAGTTGTACGTCCAGAGGCATA
Vegf	For	ACTCGGATGCCGACACGGGA
	Rev	CCTGGCCTTGCTTGCTCCCC
Mmp12	For	CAATTGGAATATGACCCCCTGT
	Rev	AGCAAGCACCCTTCACTACAT
Rpl13a	For	CATGAGGTCGGGTGGAAGTA
	Rev	GCCTGTTTCCGTAACCTCAA
Rn18s	For	GTAACCCGTTGAACCCCATT
	Rev	CCATCCAATCGGTAGTAGCG
MKI67	For	TCGACCCTACAGAGTGCTCA
	Rev	GTGGGGAGCAGAGGTTCTTC
MRC1	For	CAGATGCCCGGAGTCAGATC
	Rev	TTTATCCACAGCCACGTCCC
GAPDH	For	GATCATGAGCAATGCCTCCT
	Rev	TGTGGTCATGAGTCGTTCCA

Flow Cytometry

Single cell suspensions from left lung were prepared by digesting with 0.2 U/ml liberase TL (Roche) and 80 U/ml DNase (Life Technologies, Carlsbad, California) in HBSS at 37°C for 30 minutes. Tissue was homogenized by forcing through a 70 μM cell strainer. Cells from lung homogenates, BAL, pleural exudate, and peritoneal cavity exudate were treated with red blood cell lysis buffer (Sigma) and counted using an

automated cellometer T4 (Peqlab). Cells were incubated with Fc block (CD16/CD32 and mouse serum) and stained with fluorescent conjugated antibodies to CD19 (6D5), Siglec F (E50-2440), Ly6G (1A8), CD3 (17A2), CD45.2 (104), Ly6C (HK1.4), CD11c (N418), CD11b (M1/70), F4/80 (BM8), I-A/I-E (MHCII) (M5/114.15.), TER119 (TER-119) (eBioscience, Hatfield, UK; Biolegend, Cambridge, UK; or BD, Franklin Lakes, NJ), and unconjugated anti-Myo18A antibody (5 µg/ml) or isotype control followed by secondary reagents (Invitrogen). The following surface lineage$^-$markers identified alveolar macrophages: (CD19, Ly6G, CD3 and TER119), CD45.2$^+$, CD11c$^+$ and SiglecF$^+$. Pleural/peritoneal macrophages were identified as lineage$^-$ (CD19, SiglecF, Ly6G and CD3), CD11b$^+$ and F4/80$^+$ [5]. ILC2 cells were identified as lineage$^-$ (CD3, CD11b, CD11c, FcεR1, CD19, and NK1.1) CD90$^+$ ICOS$^+$ CD45$^+$ cells. T helper cells were identified as CD45$^+$, CD3$^+$ and CD4$^+$.

Following surface staining, cells were fixed with 2% paraformaldehyde in Dulbecco's phosphate buffered saline for 20 min at room temperature, permeabilized with Perm wash (BD), and then stained with anti-RELMα, biotinylated anti-Ym1, APC-conjugated anti-Arg-1 (R&D Systems, Minneapolis, MN), or isotype control followed by secondary reagents (Invitrogen). For detection of Ki67 and measurement of BrdU incorporation, cells were stained for surface markers, then fixed and permeabilized using FoxP3 staining buffer set (eBioscience), and subsequently stained with Ki67 set (BV56) or anti-BrdU (BU20a) for 30 min at room temperature. Cells were incubated first with or without DNase for 30 mins at 37ºC before staining with anti-BrdU antibody. For intracellular cytokine staining, lung single cell suspensions were re-stimulated ex vivo in complete RPMI1640 media (supplemented with 10% FCS, 2 mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin) containing 1 µg/ml ionomycin, 500 ng/ml PMA, and 10 µg/ml Brefeldin A for 4 hours at 37°C. Following surface staining and fixing, cells were intracellular stained with anti-IL-4 (11B11), IL-13 (eBio13A), IL-5 (TRFK5), and relevant isotypes. Expression of RELMα, Ym1, Arg-1, Ki67, IL-4, IL-13, and IL-5 was determined relative to isotype control staining. Incorporation of BrdU was determined relative to staining of non-DNase treated cells. Live/Dead (Life Technologies) was used to exclude dead cells from analysis. Samples were analysed by flow cytometry using Becton-Dickinson FACS LSR II and FlowJo software.

ELISAs

Quantification of RELMα (PeproTech, Rocky Hill, NJ) and Ym1 (R&D Systems) were performed by ELISA in BAL, pleural and peritoneal exudates, as well as in cell supernatants of *ex vivo* cultures of peritoneal or alveolar macrophages. IL-4, IL-5, IL-13, TNF-α, and IFN-γ (BioLegend and eBioscience) were measured in supernatants of *ex vivo* re-stimulated thoracic lymph node cells. C1q quantification was performed by ELISA (Source BioScience, Nottingham, UK) in peritoneal cavity exudates. ELISA assays were done following manufacturer instructions.

Isolation and culture of primary alveolar and peritoneal macrophages

Alveolar macrophages were obtained from BAL of mice and rats, and from human lung biopsies obtained from patients that were submitted to a lobectomy. An informed consent was obtained from all donors. The review board and the ethics committee of the Sabadell Hospital approved this study, which was conducted in accordance with the guidelines of the World Medical Association's Declaration of Helsinki. BAL was performed with Dulbecco's phosphate buffered saline with or without 0.2 mM EDTA and 0.5% BSA (m/v). Macrophages were purified by adherence for at least 90 min at 37°C, 5% CO2 as previously reported [313]. Adherent cells were 94.0 ± 1.1 % viable (trypan blue exclusion test). Flow cytometry analysis determined that 90 ± 1 % of adherent cells isolated from BAL were CD11c and SiglecF positive. To estimate the purity of isolated human macrophages, cells were cytospun in a CytoSpin 3 Cytocentrifuge (Shandon Scientific, Waltham, MA), and the cytospin preparations were stained with either immunostained with anti-CD68 or Diff-Quick kit (Diagnostics Grifols, Barcelona, Spain) following the manufacturer's protocol. 95 % ± 1.5 % (n=6) of adherent cells were macrophages.

Peritoneal macrophages were obtained from mice by washing the peritoneal cavity with RPMI 1640 containing 2 mM L-glutamine, 200U/ml penicillin, 100 μg/ml streptomycin. For thioglycollate-induced macrophages, mice were injected with 4% Brewer's thioglycollate (Sigma) for 48–96 h before peritoneal cells were harvested. Cells were separated from the lavage fluid by centrifugation (250 x g, 5 min), resuspended in RPMI 1640 medium (5% heat-inactivated FBS, 100 U/ml penicillin, 100 μg/ml streptomycin, supplemented with glutamine 2 mM), and purified by adherence. Viability of adherent cells was assessed by trypan blue exclusion test. Flow cytometry analysis

determined that 90 ± 1 % of adherent cell isolated from the peritoneal exudate were F4/80 and CD11b positive.

***In vitro* stimulation of alveolar and peritoneal macrophages**

Macrophages were pre-cultured for 24 h. Subsequently, cells were treated with IL-4 (0.5-1 µg/ml) (ImmunoTools, Berlin, Germany) and/or SP-A (25, 50 and 100 µg/ml) or native human C1q (10 and 100 µg/ml). The following blocking antibodies were added 2 hours before stimulation: 10 µg/ml of anti-Myo18A, 10-50 µg/ml anti-SIRPα (eBioscience,), and 10-50 µg/ml anti-calreticulin (Thermo Scientific). Under these conditions cell viability was higher than 97%. Macrophage cultures were plated in triplicate wells and each series of experiments was repeated at least three times.

Cell proliferation assays

For 5–ethynyl–2′–deoxyuridine (EdU)/BrdU incorporation analysis, cells were treated with IL-4, SP-A, C1q and combinations thereof for 24 hours. Then, cells were exposed to 10 µM EdU/BrdU for another 24 hours. For confocal microscopy analysis of EdU incorporation, cells were fixed with 2% formaldehyde for 15 minutes at room temperature and permeabilized with 0.2% saponin in PBS. EdU was detected with Alexa Fluor 647-azide using Click-iT EdU assay kit (Life Technologies). Sequential double immunostaining was performed with a monoclonal antibody to CD11c (AbD Serotec, Kidlington, UK), and immune complexes were visualized with FITC-conjugated secondary antibody (Invitrogen). Micrographs were taken with a Leica TCS SP2 Confocal System. Flow cytometry analysis of BrdU and Ki67 expression was performed as described above.

Arginase activity assay

Arginase activity was measured as previously reported [349]. Briefly, rat alveolar macrophages were lysed with 50 µl of 50 mM Tris–HCl pH 7.5, Triton X-100 0.1 %, 1 mM benzamidine, 200 µg/ml aprotinin, and 200 µg/ml leupeptin. After 30 min shaking at 4ºC, arginase was activated with 50 µl of 10 mM $MnCl_2$ and 50 mM Tris-HCl, pH 7.5, for 10 min at 55º C. L-arginine hydrolysis was measured by incubating the cell lysate with 25 µl of 0.5 M L-arginine (Sigma) (pH 9.7) at 37ºC for 1 h. The reaction was stopped by addition of 200 µl H_2SO_4 /H_3PO_4 /H_2O (1:3:7 v/v). The produced urea was quantified at 570 nm after addition of 25 µl of α-isonitrosopropiophenone (dissolved in

100% ethanol) followed by heating at 99ºC for 45 min. Urea production was normalized to cell number for each treatment by quantifying cells with the WST-1 reagent (Roche), following manufacturer' instructions. One unit of arginase activity is defined as the amount of enzyme that catalyses the formation of 1 µmol urea per min.

siRNA-mediated gene-silencing effects *in vitro*

After isolation, primary alveolar macrophages were resuspended in Amaxa® mouse macrophage nucleofector solution (Lonza) and nucleofected with 100 nM siRNA using a nucleofector 2b device (Lonza). Experiments were conducted using two Stealth siRNAs directed against rat Myo18A (RSS322720 and RSS322721) (Applied Biosystems, Carlsbad, California). Medium GC Stealth siRNA was used as control (12935300) (Applied Biosystems). Myo18A expression was detected by Western blot analysis with an anti-Myo18A antibody. After 48 hours post nucleofection, Myo18A expression was reduced 72 ± 4% for RSS322720 and 71 ± 5% for RSS322721 compared to control. At this time-point, cells were stimulated.

Statistics

Normal distribution of data was determined by visual examination of residuals. Statistical evaluation of different groups was performed either by analysis of variance (ANOVA) followed by the Bonferroni multiple comparison test or by unpaired two-tailed Student's t-test, as indicated. An α level ≤ 5% ($p \leq 0.05$) was considered significant. All statistical calculations were performed using PRISM, (Graphpad La Jolla, CA).

Results

SP-A limits helminth infection and contributes to lung repair

To examine the role of SP-A during helminth-induced type 2 immune responses in the lung, we inoculated WT, IL-4Rα-deficient and SP-A-deficient mice with 250 *N brasiliensis* infective larvae. Larvae enter the lung between days 1 and 2 post inoculation, mature in the lung for ~2 days and migrate to the small intestine by day 3. The type 2 response peaks at days 6-7 after inoculation [350]. We observed an up-regulation of SP-A (mRNA and protein) in lungs of *N brasiliensis* infected C57BL/6 mice at day 6 (**Fig. 1a**). Analysis of SP-A mRNA and protein in lungs of IL-4Rα$^{-/-}$ mice showed that helminth-induced SP-A up-regulation was dependent on IL-4 and/or IL-13 (**Fig. 1a**).

Consistent with a role for SP-A during type 2 immunity to *N brasiliensis* infection, SP-A-deficient mice had increased adult worm burden (**Fig. 1b**) and egg output (**Fig. 1c**) when infected with either 250 or 400 infective third-stage larvae. In addition, the absence of SP-A significantly impaired the lung repair process as indicated by loss of alveolar surface area. (**Fig.1d,e**). Infected SP-A-deficient mice also exhibited increased expression of *Mmp12* mRNA (Mmp-12) relative to infected WT mice (**Fig. 1f**). This likely reflects increased inflammatory macrophage infiltration, consistent with the known role for SP-A in reducing inflammation associated with lung injury [336]. Finally, *N brasiliensis*-infected WT mice, but not SP-A$^{-/-}$ mice, showed increased expression of the tissue-repair related gene *Col1a1* (Collagen, type I, alpha 1) (**Fig. 1g**)

Although there were no major differences between WT and SP-A-deficient mice in the cellular composition of the bronchoalveolar lavage (BAL), lung homogenates, or pleural lavage (data not shown), SP-A-deficient mice exhibited reduced protein expression of the M(IL-4) markers RELMα, Ym1, and Arginase (Arg1) in aMϕs isolated from BAL (**Fig. 1h**) and lungs **(Extended Fig. 1a)**. Consistent with intracellular flow cytometry analysis, secretion of RELMα and Ym1 protein to the alveolar fluid (**Fig. 1i**) and pleural space (**Fig. 1j**) was enhanced at 6 days post infection in both WT and SP-A-deficient mice, but reduced in SP-A$^{-/-}$ mice compared to WT mice. In addition to the induction of M(IL-4) markers, IL-4 typically induces macrophage proliferation during helminth infection [351]. At day 6 following infection, proliferation of aMϕs from BAL (**Fig. 1k-m**) and lung tissue (**Extended Fig. 1b**) of WT mice was observed, as

measured by cell number (**Fig. 1k**), expression of the nuclear cell-cycle antigen Ki67 **(Fig. 1l and Extended Fig. 1b),** and BrdU incorporation (**Fig. 1m and Extended Fig. 1b**). In comparison to their WT littermates, SP-A-deficient mice failed to exhibit significantly enhanced macrophage proliferation in response to *N brasiliensis* infection in BAL (**Fig. 1k-m**) and lung (**Extended Fig. 1b**).

To explore whether SP-A's effects on worm burden and wound repair might occur via promotion of adaptive T_H2 responses, we analyzed cytokines secreted by lung-draining lymph node cells re-stimulated with parasite-antigens. Samples from SP-A-deficient mice showed a slight increase of IL-4, IL-5, IL-13, TNF-α, and IFN-γ (**Extended Fig. 1c**), perhaps due to enhanced dendritic cell maturation and therefore T cell activation in SP-A-deficient mice [302]. However, we saw no evidence of any difference in total numbers of ILC2 and $CD4^+$ lung resident cells or in their ability to produce IL-4, IL-13, and IL-5 (**Extended Fig. 1d**). Similarly, the measurement of *Il13, Il5*, and *Tnf* mRNA in lung homogenates suggested that both mouse strains induce a similar cytokine profile after helminth infection (**Extended Fig. 1e**).

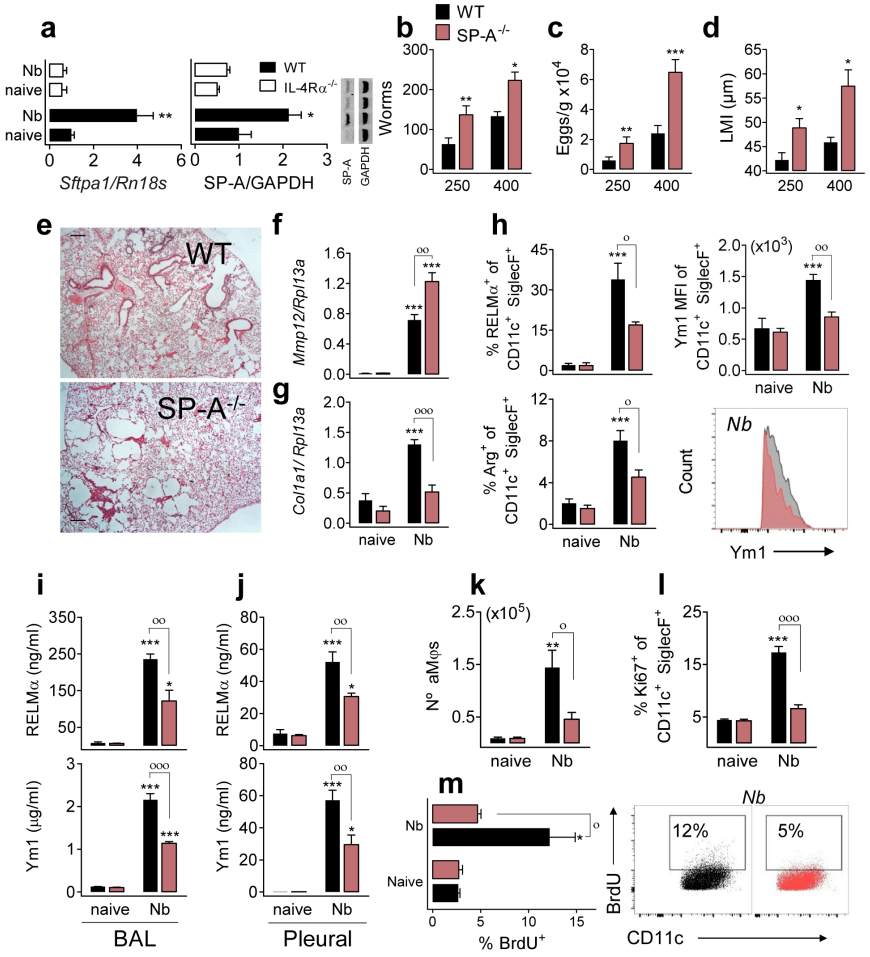
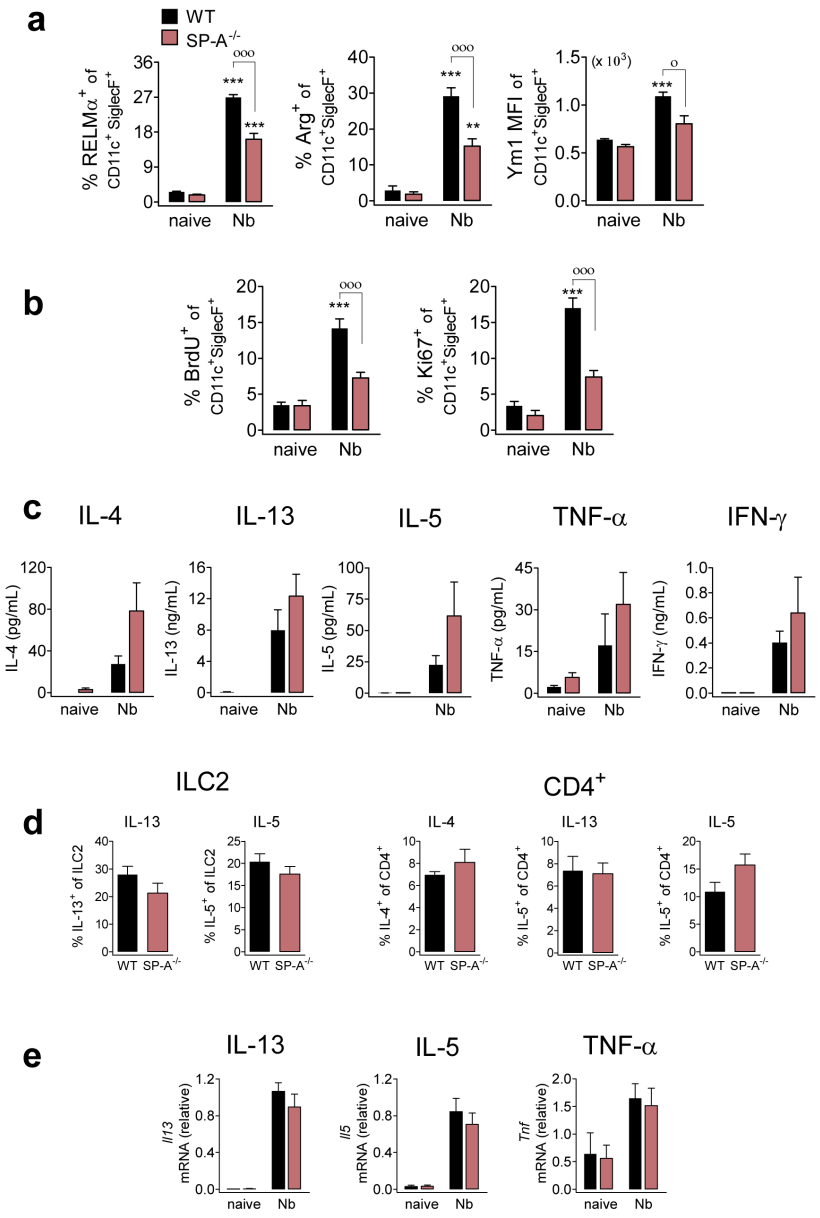


Figure 1. *Nippostrongylus brasiliensis* infected SP-A$^{-/-}$ mice have higher worm burdens and greater nematode-induced lung damage. WT, SP-A$^{-/-}$, or IL-4Rα$^{-/-}$ mice were left uninfected or infected with *N. brasiliensis* L3's, and samples were assessed at day 6 after infection. **(a)** SP-A mRNA (normalized by *Rn18s*) and protein expression in lung tissue of WT and IL-4Rα$^{-/-}$ mice. **(b)** Adult larvae in the small intestine. **(c)** Egg output in faeces. **(d)** Quantification of lung damage, calculated as 'linear means intercept' from **(e)** microscopy of H&E stained lung sections (scale bars, 500 μm). Amplification of **(f)** *Mmp12* and (**g**) *Col1a1*-encoding mRNA in lung tissue. **(h)** Expression of RELMα, Ym1, and Arg by alveolar macrophages from BAL. Because all aMϕ are Ym1 positive, MFI is shown for Ym1 rather than % positive; A representative histogram of Ym1 from WT and SP-A$^{-/-}$ infected animals is shown. Levels of RELMα and Ym1 in **(i)** alveolar and **(j)** pleural spaces. **(k)** Number of alveolar macrophages recovered in BAL. Expression of **(l)** Ki67 and **(m)** BrdU incorporation by alveolar macrophages from BAL; a representative plot showing BrdU staining in macrophages from WT and SP-A$^{-/-}$ infected animals is shown. Numbers by outlined areas indicate percent of BrdU^{+} macrophages. Data are representative from two independent experiments (mean ± SEM; naïve: 3 mice, *Nb*: 6 mice). ANOVA followed by the Bonferroni multiple-comparison test or Student's *t*-test (b-d) was used. *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$, when compared with the uninfected group; °$p < 0.05$, °°$p < 0.01$, and °°°$p < 0.001$ when WT vs. SP-A$^{-/-}$ infected groups are compared.



Extended Figure 1. Further characterization of WT vs. SP-A-deficient mice during *Nippostrongylus brasiliensis* infection. WT or SP-A$^{-/-}$ mice were left uninfected or infected with 200 *N. brasiliensis* L3's, and assessed at day 6 after infection. (a) Expression of the M(IL-4) markers RELMα, Ym1, and arginase (Arg), in alveolar macrophages from single-cell suspensions of lung tissue. (b) Proliferation of alveolar macrophages from lung tissue, measured by BrdU incorporation and Ki67 expression. (c) Levels of IL-4, IL-13, IL-5, TNFα, and IFN-γ in supernatants of thoracic lymph node cells from WT and SP-A$^{-/-}$ mice, cultured with *N. brasiliensis* antigen (1 μg/ml); results are normalized to those obtained for cells cultured with medium alone. (d) Expression of IL-4, IL-13 and IL-5 by ILC2 and CD4+ cells from single-cell suspensions of lung tissue stimulated ex vivo with PMA and ionomycin. (e) Amplification of *Il13*, *Il5* and *Tnf*-encoding mRNA in lung homogenates is also shown. Results are representative from two independent experiments (means ± SEM) (naïve: 3 mice, Nb: 6 mice). ANOVA followed by the Bonferroni multiple-comparison test (a-c) or Student's t-test (d) was used. **$p < 0.01$ and ***$p < 0.001$, when compared with the uninfected group; °$p < 0.05$ and °°°$p < 0.001$ when WT vs. SP-A$^{-/-}$ infected groups are compared.

SP-A enhances IL-4-induced proliferation and activation of alveolar macrophages

We chose next to address whether the observed failure of SP-A-deficient mice to exhibit full M(IL-4) activation and proliferation was because SP-A enhances IL-4Rα-mediated effects on aMϕs. We thus injected WT and SP-A-deficient mice *i.p.* with IL-4 complex (IL-4c) and assessed macrophage proliferation as described previously [102]. Consistent with our previous report [102] Mϕs that exhibit high Ki67 expression directly correspond to Mϕs positive for BrdU following a 3hr BrU pulse (**Fig. 2a**). Thus $Ki67^{high}/BrdU^{+}$ cells represented the subpopulation of aMϕs that were actively dividing upon stimulation. Delivery of IL-4c induced proliferation of aMϕs isolated from BAL (**Fig. 2a**) and lungs (**Extended Fig. 2a**) of WT mice but not SP-A-deficient mice. Consistent with the lack of IL-4c-induced proliferation, SP-A-deficient mice showed reduced IL-4c dependent M(IL-4) markers in both BAL (**Fig. 2b,c**) and lung (**Extended Fig. 2b**) aMϕs, as measured by intracellular expression and secretion of RELMα and Ym1 into the alveolar fluid (**Fig. 2b,c**).

Up-regulation of the IL-4Rα could explain SP-A's ability to enhance IL-4-induced effects. However, we observed no differences in IL-4Rα expression between WT and SP-A-deficient aMϕs following IL-4c treatment (**Fig. 2d**). Peritoneal delivery of IL-4c was sufficient to increase the amount of SP-A protein in BAL and lungs (**Fig. 2e**), confirming that the up-regulation of SP-A observed in the helminth infection model (**Fig. 1a**) was at least in part, due to the type 2 response.

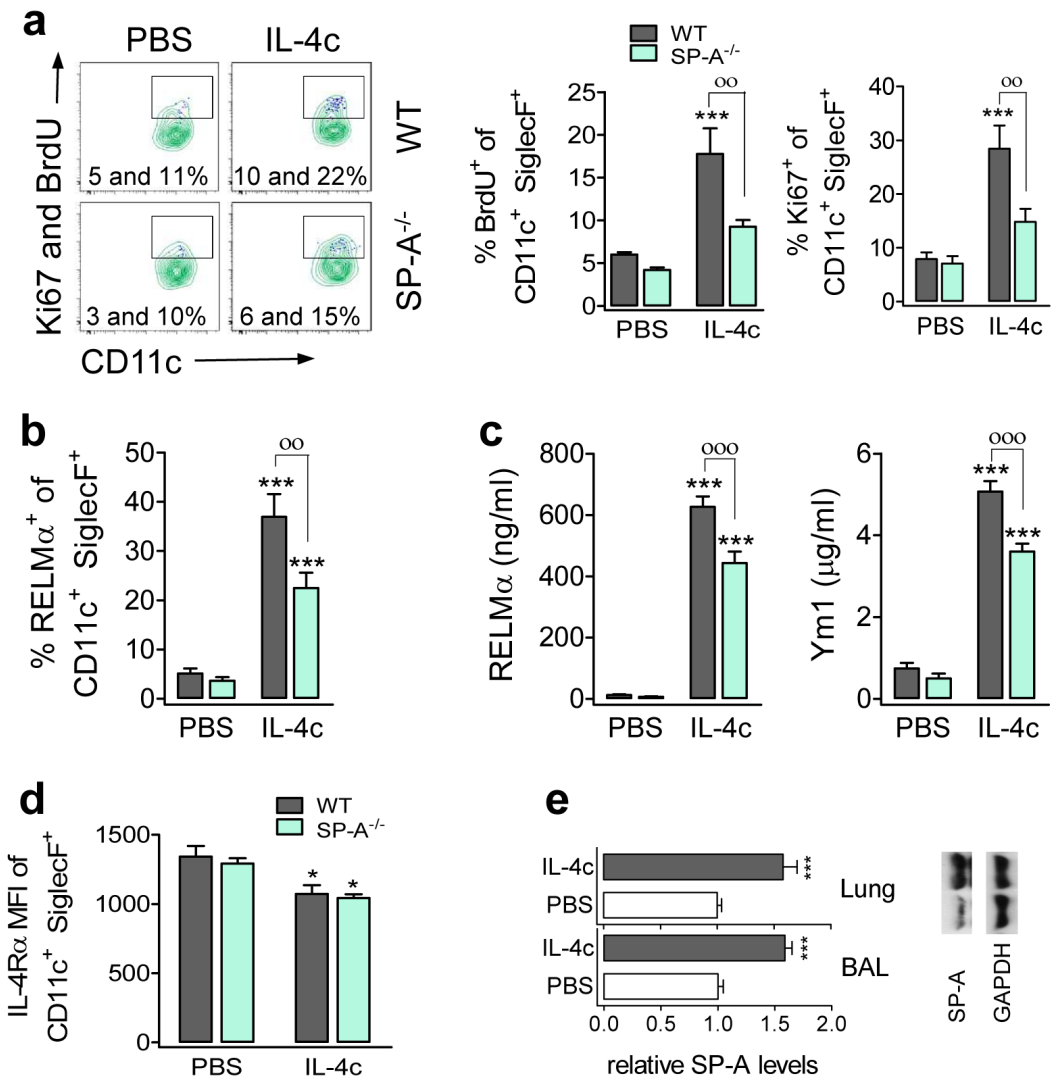
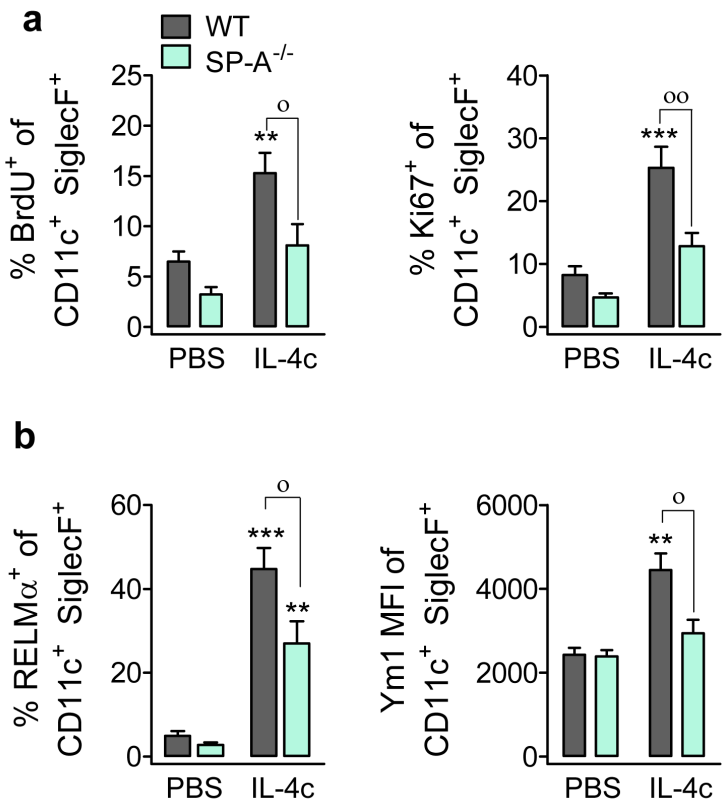


Figure 2. Alveolar macrophages from SP-A$^{-/-}$ mice show decreased IL-4-induced proliferation and alternative activation. WT and SP-A$^{-/-}$ mice were treated with 5μg of IL-4c (i.p.) at days 0 and 2; samples were analyzed at day 4. **(a)** Expression of Ki67 and BrdU incorporation by alveolar macrophages from BAL. A representative plot from WT and SP-A$^{-/-}$ mice treated with PBS or IL-4c is shown. Ki67 (green contour) and BrdU^{+} cells are overlaid as blue dots. Numbers below outlined areas indicate percent of BrdU^{+} and Ki67^{+} macrophages. **(b)** Expression of RELMα by alveolar macrophages from BAL. **(c)** Analysis of secreted RELMα and Ym1 from BAL. **(d)** Expression of IL-4Rα by alveolar macrophages from BAL. **(e)** SP-A expression levels in BAL and lung tissue (a representative Western Blot analysis from lung homogenates is shown). Data were pooled from three independent experiments (means ± SEM) (PBS: 9 mice, IL-4c: 11 mice). ANOVA followed by the Bonferroni multiple-comparison test or unpaired two-tailed Student's *t*-test (e) was used. *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$ when compared with the untreated group. °°$p < 0.01$ and °°°$p < 0.001$ when WT vs. SP-A$^{-/-}$ groups are compared.



Extended Figure 2. Proliferation and activation of alveolar macrophages from lung tissue of WT and SP-A-/- mice in response to IL-4c. WT and SP-A$^{-/-}$ mice were treated with 5μg of IL-4c (i.p.) at days 0 and 2; samples were analyzed at day 4. (a) Proliferation of alveolar macrophages from lung tissue in response to IL-4c. (b) Expression of the M(IL-4) markers RELMα and Ym1 in alveolar macrophages from lung tissue. Data were pooled from three independent experiments (means ± SEM) (PBS: 9 mice, IL-4c: 11 mice). ANOVA followed by the Bonferroni multiple-comparison test was used. **$p < 0.01$ and ***$p < 0.001$ when compared with the untreated group. °$p < 0.05$ and °°$p < 0.01$ when WT vs. SP-A$^{-/-}$ groups are compared.

SP-A boosts IL-4 effects on alveolar, but not peritoneal, macrophages

To determine whether SP-A acts directly and specifically on alveolar macrophages, adherence purified macrophages from the alveolar and peritoneal spaces were treated with 1 μg/ml of IL-4 and the ability of SP-A to enhance proliferation or activation assessed. C1q was chosen as a control because it is a defense collagen structurally homologous to SP-A [11, 338]. In response to IL-4 alone aMϕ, but not resident peritoneal (pMϕ) or thioglycollate elicited macrophages (Thio-Mϕ) proliferated *in vitro* (**Fig. 3a**). Notably, cultured aMϕ did not significantly increase RELMα expression in response to IL-4 but were Ym1^{+} regardless of IL-4 treatment (**Fig. 3a**). In contrast pMϕ upregulated both RELMα and Ym1 following *in vitro* treatment with IL-4.

SP-A, but not C1q, significantly boosted IL-4-mediated aMϕ but not pMϕ proliferation and M(IL-4) markers (**Fig. 3b**). Alveolar macrophages from IL-4Rα-deficient mice showed no proliferation or activation when stimulated with IL-4 (1 μg/ml) in the absence or presence of SP-A (**Fig. 3c**). To our surprise C1q, but not SP-A, significantly increased IL-4-mediated pMϕ but not aMϕ proliferation (**Fig. 3b**). In addition, C1q significantly enhanced IL-4 dependent secretion of Ym1, as assessed by ELISA analysis of pMϕ culture supernatant (**Fig. 3b**). Thus SP-A acts as a tissue-specific factor to directly enhance IL-4-mediated activation and proliferation of alveolar, but not peritoneal, macrophages, and C1q appears to have the same function for peritoneal cells.

Having established this *in vitro* system, we were able to ask whether SP-A's enhancement of IL-4 could be observed in humans. Human aMϕs were isolated from macroscopically normal tissue biopsies from patients with lung carcinoma. Isolated human aMϕs were cultured in the presence of recombinant human IL-4 (0.5 μg/mL) with or without SP-A for 48 hours. We found that *in vitro* treatment of human aMϕs with IL-4 was sufficient to increase the mRNA level of *MKI67* and *MRC1* as markers of proliferation and M(IL-4), respectively (**Fig. 3d**). Similarly, SP-A significantly enhanced proliferation and activation induced by IL-4 in rat aMϕs (**Fig. 3e**).

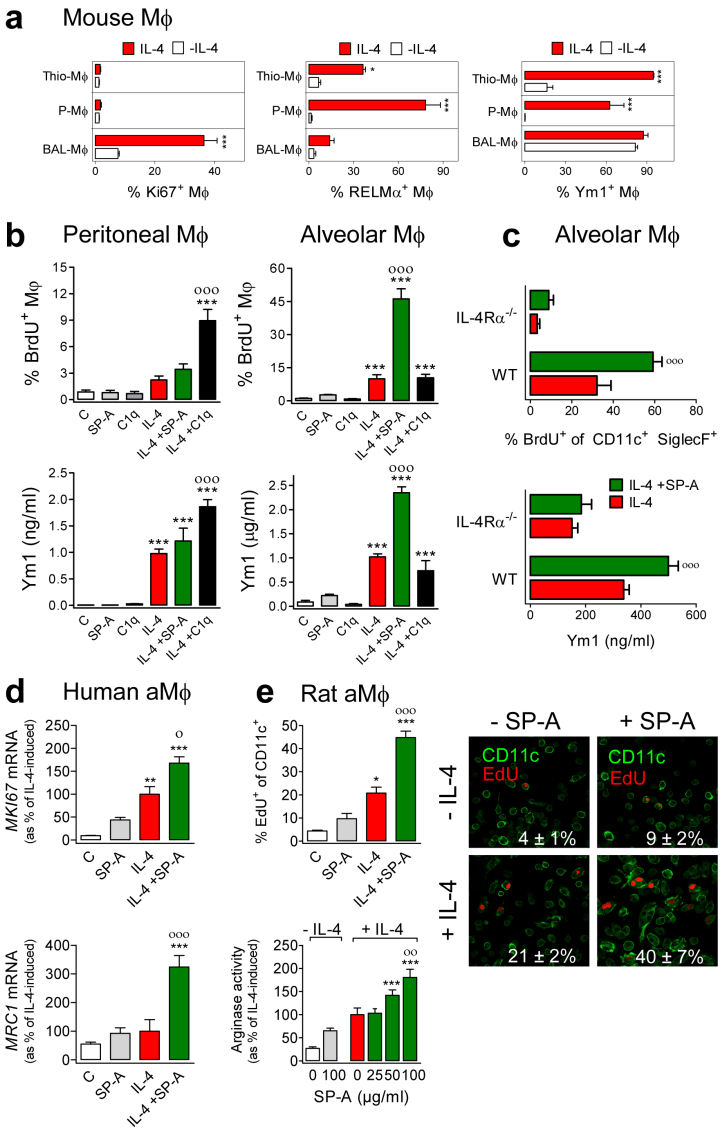


Figure 3. SP-**A and C1q enhance** IL-4-induced proliferation and activation of **alveolar and peritoneal macrophages, respectively.** Purified alveolar and peritoneal (resident and thioglycollate-recruited) from mice (a-c), humans (d), or rats (e) were treated with or without IL-4 (0.5-1 µg/ml) in the presence or absence of SP-A or C1q and exposed to BrdU for proliferation analysis. **(a)** Ki67, RELMα, and Ym1 expression by aMϕ and pMϕ (resident and thioglycollate-recruited) in the absence of either SP-A or C1q. (**b**) BrdU incorporation and secretion of Ym1 by resident peritoneal and alveolar macrophages. **(c)** BrdU incorporation and secretion of Ym1 by cultured alveolar macrophages from WT and IL-4Rα$^{-/-}$ mice. **(d)** *MKI67* and *MRC1* mRNA expression by human alveolar macrophages as measured by qRT-PCR. (**e**) EdU incorporation analyzed by flow cytometry and arginase activity of rat alveolar macrophages. A representative confocal micrograph of rat alveolar macrophages immuno-stained with CD11c-FITC and EdU-Alexa Fluor 647 is shown. Numbers below indicate percentage ± SEM of EdU^{+} cells from three independent experiments. The results are presented as means (± SEM) from three different cell cultures with at least three biological replicates. ANOVA followed by the Bonferroni multiple-comparison test was used. *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$, when compared with untreated macrophages (C); $^{o}p < 0.05$, $^{oo}p < 0.01$, and $^{ooo}p < 0.001$, when either SP-A+IL-4- or C1q+IL4-treated macrophages were compared with IL-4-treated macrophages.

Myo18A mediates the effects of SP-A and C1q on alveolar and peritoneal macrophages, respectively

To determine which SP-A receptor is mediating SP-A effects on IL-4-stimulated aMϕs, we inhibited known receptors for SP-A: signal inhibitory regulatory protein α (SIRPα), calreticulin (*aka* cC1qR), and Myo18A (*aka* SP-R210) [11]. We observed that the blockade of Myo18A, but not of calreticulin or SIRPα receptors abrogated SP-A-mediated enhancement of IL-4-induced arginase activity in rat aMϕs (**Extended Fig. 4a**). RNA silencing of Myo18A consistently abolished SP-A-mediated enhancement of IL-4-induced proliferation and activation of rat aMϕs (**Fig. 4a**). Human aMϕs also showed significant reduction of *MKI67* and *MRC1* mRNA expression in the presence of anti-Myo18A antibody (**Fig. 4b**), confirming that SP-A must bind to Myo18A receptor to enhance proliferation and alternative activation of human aMϕs.

Myo18A is an unconventional myosin that does not operate as a traditional molecular motor in cells, having both intracellular and cell-surface locations [352]. Immune activation results in Myo18A localization on the cell surface, where it binds to SP-A with high affinity and thus is also known as SP-R210[291, 338]. The collagen-like domain of SP-A is essential for functional interaction with Myo18A [338, 353]. We therefore examined the role of the SP-A collagen-like tail for Myo18A receptor signaling by comparing the effects of native SP-A and recombinant human SP-A1 expressed in insect cells (SP-A1^{hyp}). SP-A1^{hyp} lacks the prolyl hydroxylation in the collagen domain, which results in improper folding [340]. We found that SP-A1^{hyp} had no effect on IL-4-mediated proliferation and activation of rat aMϕs (**Extended Fig. 4b**) but still functioned to kill bacteria like native SP-A (**Extended Fig. 4c**). These results suggest that an intact collagen-like domain is required to enhance IL-4-mediated type 2 responses via Myo18A receptor.

The role of the collagen-like tail was further supported by our finding that C1q, structurally homologous to SP-A in its supra-trimeric assembly and collagen tail [11, 338], enhanced IL-4 driven activation and proliferation of mouse peritoneal Mϕ via Myo18A (**Fig. 4c**). Blockade of Myo18A also abrogated SP-A effects on mouse aMϕs (**Fig. 4d)**. Notably, we observed that IL-4 promotes Myo18A localization on the cell

surface of peritoneal (**Fig. 4e**) and alveolar (**Fig. 4f**) Mϕs after 24 hour of IL-4 stimulation. Expression of Myo18A on the cell surface was greater for alveolar than peritoneal Mϕs nicely matching the greater cellular response to IL-4+SP-A than IL-4+C1q **(Fig. 4c,d).**

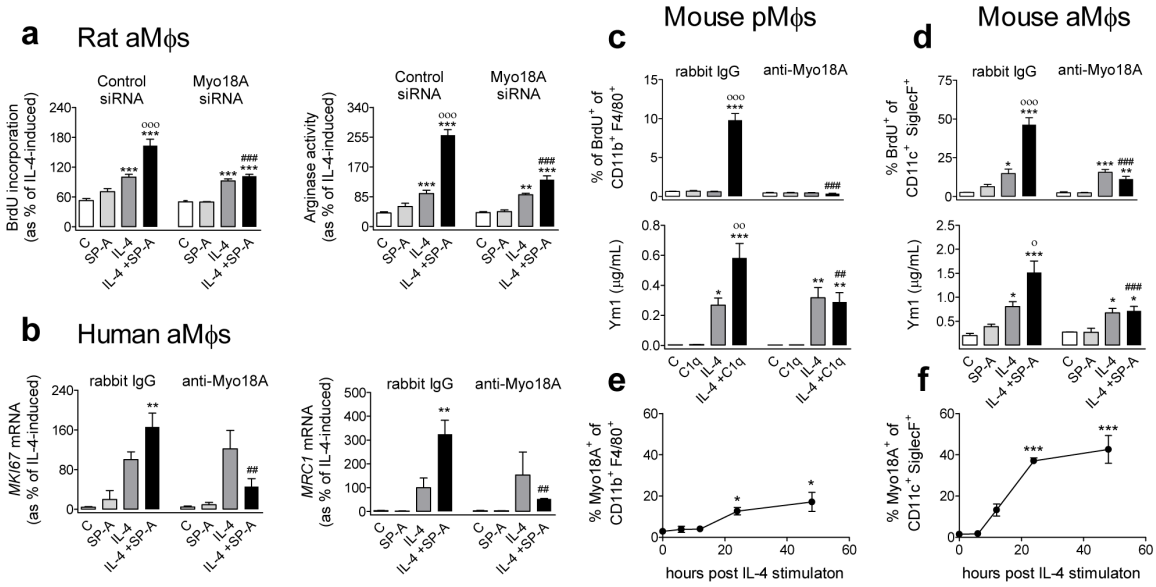
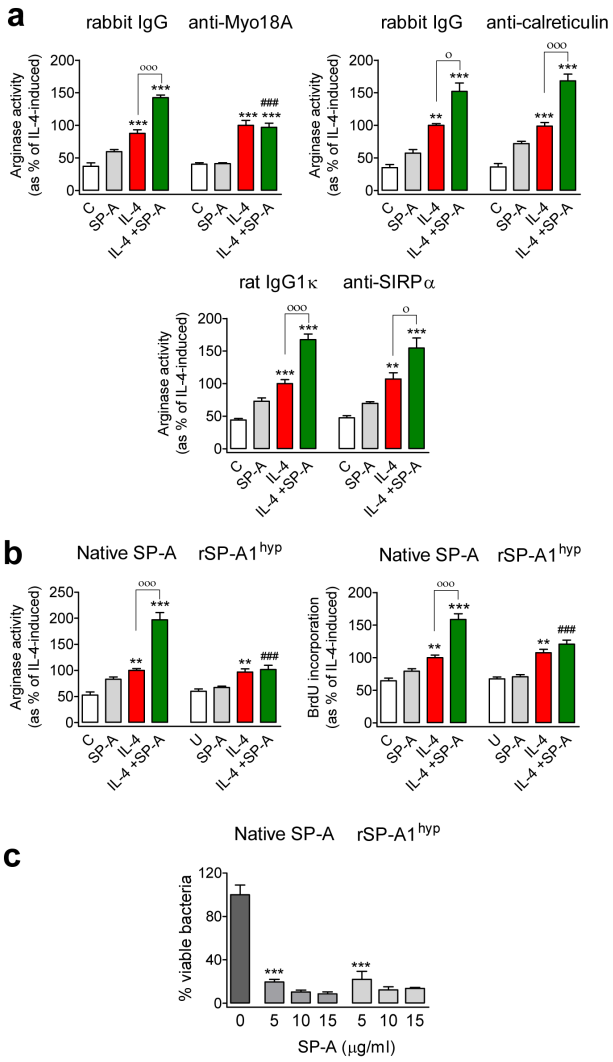


Figure 4. Myo18A mediates the effects of SP-A and C1q on alveolar and peritoneal macrophages, respectively. Isolated rat, human, and mouse alveolar macrophages as well as purified resident peritoneal macrophages from mice were nucleofected with Myo18A siRNA (RSS322720) or control siRNA, pre-treated with 10 μg/ml of anti-Myo18A neutralizing antibody or an IgG control for two hours, or untreated. Next, cells were stimulated with or without IL-4 (0.5-1 μg/ml) in the presence or absence of either SP-A or C1q and exposed to BrdU for proliferation analysis. **(a)** BrdU incorporation and arginase activity in rat alveolar macrophages that were nucleofected with Myo18A (RSS322720) or control siRNA. Similar results were found using RSS322721 Myo18A siRNA. **(b)** mRNA expression of *MKI67* and *MRC1* by human alveolar macrophages treated with anti-Myo18A antibody. **(c,d)** Anti-Myo18A blockade of proliferation and activation of mouse macrophages treated with either **(c)** IL-4+C1q (pMϕ) or **(d)** IL-4+SP-A (aMϕ). **(e,f)** Time-dependent expression of Myo18A on the cell surface of peritoneal **(e)** and alveolar **(f)** macrophages in response to IL-4 treatment. The data shown are means ± SEM of three different alveolar or peritoneal macrophage cultures with at least three biological replicates. ANOVA followed by the Bonferroni multiple-comparison test was used. *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$, when compared with untreated cells (C); °°$p < 0.01$, and °°°$p < 0.001$, when either SP-A+IL-4- or C1q+IL4-treated macrophages were compared with IL-4-treated macrophages; #$p < 0.05$, ##$p < 0.01$, and ###$p < 0.001$, when the effect of either Myo18A siRNA or anti-Myo18A antibody is compared in cells treated with SP-A+IL-4 or C1q+IL4.



Extended Figure 4. (a) Myo18A, but not calreticulin or SIRPα, mediates SP-A's effects on aMφs stimulated with IL-4. Purified rat alveolar macrophages were treated with either anti-Myo18A (10 μg/ml), anti-calreticulin (10-50 μg/ml), anti-SIRPα (10-50 μg/ml), or an isotype control for two hours. Next, cells were IL-4-stimulated with or without native SP-A. **(b) Requirement of the collagen-like domain of SP-A for functional interaction with Myo18A.** Purified rat alveolar macrophages were treated with anti-Myo18A (10 μg/ml) for two hours and then cells were IL-4-stimulated with or without either native human SP-A or recombinant human SP-A1 expressed in insect cells (SP-A1hyp). Arginase activity and proliferation (BrdU incorporation) were measured. **SP-A1hyp exhibits an improper collagen domain folding but still functions to kill E.coli J5 bacteria (105 CFUs/ml) like native SP-A (c).** The survival of bacteria in the presence of SP-A is shown as a percentage of the number of colonies of the same strain not exposed to SP-A. The data shown are means ± SEM of three different alveolar macrophage cultures with at least three biological replicates. ANOVA followed by the Bonferroni multiple-comparison test was used. **p < 0.01 and ***p < 0.001, when compared with untreated cells; °°p < 0.01 and °°°p < 0.001, when SPA+ IL-4-treated Mφs are compared with IL-4-treated Mφs; ###p < 0.001, when either the effect of anti- Myo18A antibody vs. rabbit IgG is compared or when the effects of native SP-A vs. SP-A1hyp on arginase activity are compared.

Myo18A blockade reduces IL-4-mediated activation and proliferation of macrophages *in vivo*

We next confirmed the role of Myo18A *in vivo.* Delivery of IL-4c *i.p.* induced the expression of Myo18A on the cell surface of both aMϕ and pMϕ, which was independent of the presence or absence of SP-A or C1q, respectively (**Fig. 5a,b**). Antibody blockade of Myo18A phenocopied SP-A deficiency. In both settings, aMϕs exhibited little proliferation or expression of M(IL-4) markers in response to IL-4 (**Fig. 5a**). Similarly, the secretion of RELMα and Ym1 to the alveolar fluid was highly reduced by the blocking antibody (**Extended Fig. 5a**). Delivery of anti-Myo18A antibody also reduced IL-4c-induced proliferation and activation of pMϕs (**Fig. 5b)** and secretion of RELMα and Ym1 to the peritoneal cavity (**Extended Fig. 5b**). Taken together, these data suggest that Myo18A is a common receptor or co-receptor for defense collagens present on alveolar and peritoneal Mϕs, which helps determine their capacity to respond to IL-4. Given that Myo18A lacks a trans-membrane domain [352], transmembrane co-receptors must act in concert with Myo18A for signal transduction. Tissue-specificity would then be imparted by these coreceptors whose expression would be determined by cell origin or local imprinting by the tissue environment.

C1qa-deficient mice show reduced IL-4-mediated type 2 responses

Similar to SP-A in the lung **(Fig. 2e),** C1q levels increased in the peritoneal fluid after IL-4c delivery (**Fig. 5c**), consistent with the reported expression of C1q by M(IL-4) [354]. We thus evaluated the effect of C1q on IL-4-mediated type 2 responses. In contrast to WT mice, pMϕs from C1qa-deficient mice had significantly lower levels of proliferation and M(IL-4) marker expression following IL-4c delivery (**Fig. 5d & Extended Fig. 5c**). These data show that C1q acts to enhance IL-4 mediated Mϕ proliferation and activation in the peritoneal cavity.

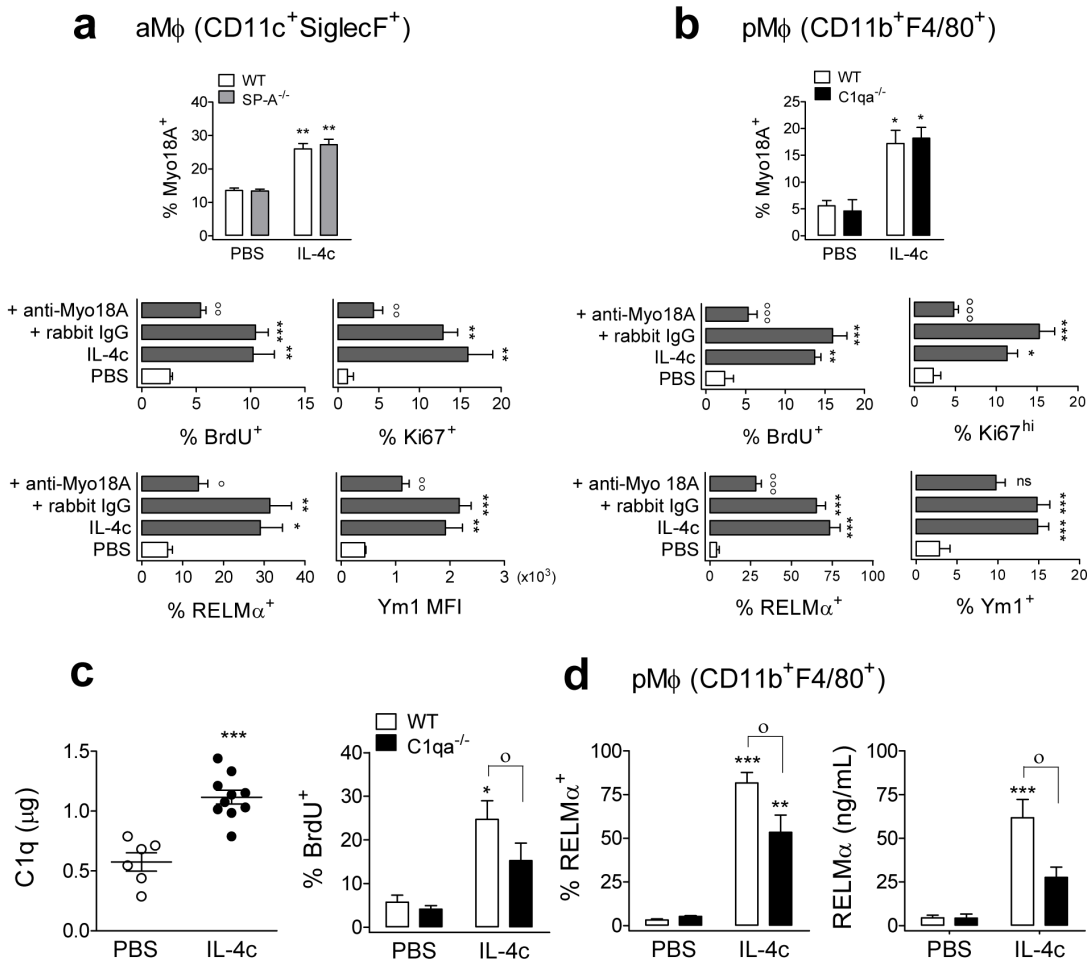
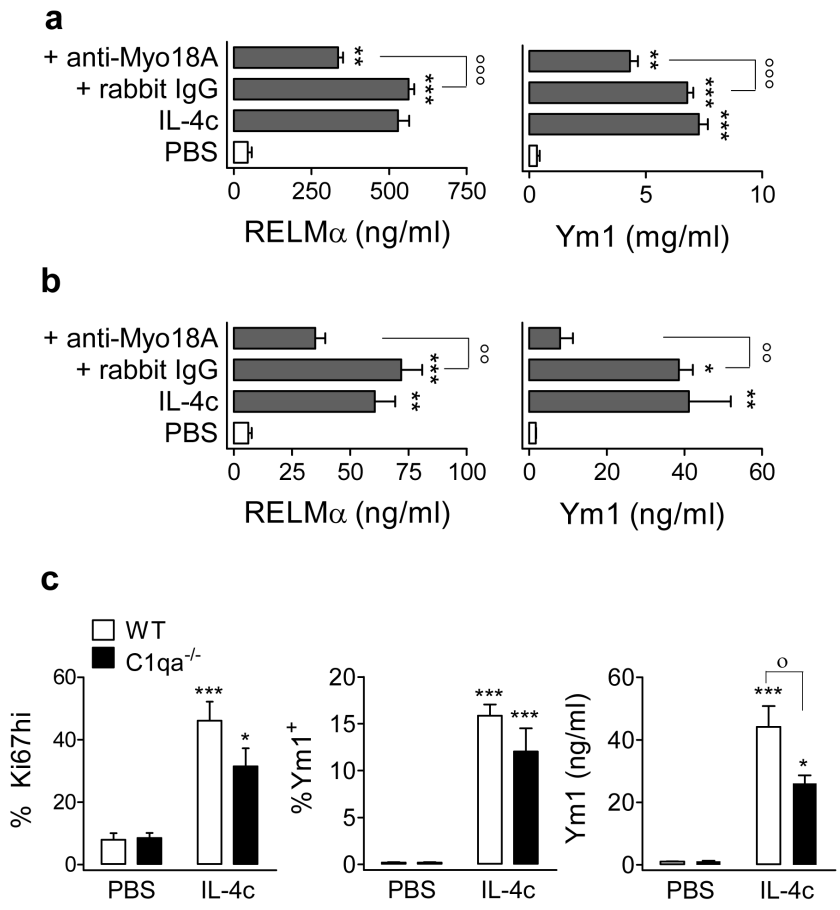


Figure 5. (a, b) Antibodies against Myo18A blocked IL-4-mediated activation and proliferation of macrophages *in vivo*. (a) For aMϕ analysis, WT or SP-A$^{-/-}$ mice received 5 μg of IL-4c (*i.p.*) at day 0 and 2. Simultaneously, animals were intra-nasally treated with either anti-Myo18A or rabbit IgG antibody at day 2 and 3, and samples analyzed at day 4. IL-4-mediated proliferation (% BrdU^{+} and % Ki67^{+}) and activation (% RELMα^{+} and Ym1) of aMϕs is shown. (b) For pMϕ analysis, WT or C1qa$^{-/-}$ mice received 1 μg of IL-4c (i.p.) at day 0, and samples were analyzed at day 1. Some mice were also treated with either anti-Myo18A or rabbit IgG antibody (*i.p.*) 2 hours before IL-4c administration. IL-4-mediated proliferation (% BrdU^{+} and % Ki67^{+}) and activation (% RELMα^{+} and %Ym1^{+}) of pMϕs are shown. **(c) C1q protein levels in the peritoneal cavity:** Following IL-4c delivery, C1q protein was measured by ELISA in WT mice. **(d) C1qa-deficient mice show reduced IL-4-mediated type 2 responses:** IL-4-mediated proliferation (% BrdU^{+} and % Ki67^{+}) and activation (% RELMα^{+}) of pMϕs in WT vs C1qa$^{-/-}$ mice; RELMα secretion into the peritoneal cavity was measured by ELISA. Data were pooled from three independent experiments (means ± SEM) (PBS: 6 mice, other groups: 9 mice). ANOVA followed by the Bonferroni multiple-comparison test was used. *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$, when compared with PBS-treated mice; °$p < 0.05$, °°$p < 0.01$, and °°°$p < 0.001$ when either WT vs. C1qa$^{-/-}$ mice treated with IL-4c are compared or when anti-Myo18A vs rabbit IgG treatment is compared in IL-4c-treated mice.



Extended Figure 5. Antibodies against Myo18A reduced IL-4-mediated secretion of RELMα and Ym1 to the alveolar (a) and peritoneal (b) cavities. (a) For alveolar macrophage analysis, WT or SP-A$^{-/-}$ mice received 5 µg of IL-4c (i.p.) at day 0 and 2. Simultaneously, these animals were intra-nasally treated with either anti-Myo18A or rabbit IgG antibody at day 2 and 3, and samples were analyzed at day 4. (b) For peritoneal macrophage analysis, WT or C1qa$^{-/-}$ mice received 1 µg of IL-4c (i.p.) at day 0, and samples were analyzed at day 1. Mice were treated with either anti-Myo18A or rabbit IgG antibody (i.p.) 2 hours before IL-4c administration. **(c) Measurement of Ki67 and Ym1 expression in WT vs C1qa$^{-/-}$ following IL-4c delivery.** Data were pooled from three independent experiments (means ± SEM) (PBS: 6 mice, other groups: 9 mice). ANOVA followed by the Bonferroni multiple-comparison test was used. *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$, when compared with PBS-treated mice; °°$p < 0.01$ and °°°$p < 0.001$ when either anti-Myo18A vs rabbit IgG treatment (a,b) or WT vs. C1qa$^{-/-}$ mice (c) are compared in IL-4ctreated mice.

C1q-dependent enhancement of type 2 responses in a model of peritoneal fibrosis

To ascertain the physiological relevance of C1q-dependent enhancement of type 2 responses, we evaluated the role of C1q in a murine model of peritoneal fibrosis induced by Dianeal-PD4, a lactate dialysate used in the clinic [346]. Peritoneal fibrosis is a frequent and serious consequence of peritoneal dialysis [355] and is associated with M(IL-4) in both humans and mice [346, 355, 356]. In our model, lactic acid could be responsible for the induction of macrophage proliferation and Mϕ2 activation [357]. We administered Dianeal-PD4 every other day for 28 days to WT and C1qa-deficient mice. In WT but not in C1qa-deficient mice, Dianeal-PD4 treatment provoked the induction of C1q (**Fig. 6a**) and morphologic changes in tissue sections of the parietal peritoneum, showing significant enlargement of the submesothelial zone caused by collagen deposition (**Fig. 6b,c**). Dianeal-PD4 treatment also induced markers of fibrosis including collagen mRNAs (*Col1a1* and *Col3a1*) (**Fig. 6d**), alpha-smooth muscle actin (*Acta2)* **(Fig. 6e),** and vascular endothelial growth factor *(Vegf)* **(Fig. 6f).** Importantly, significant upregulation of these markers was not observed in C1qa-deficient mice (**Fig. 6d-f**). Conversely, *Mmp12* mRNA was up-regulated in C1qa-deficient relative to WT mice **(Fig. 6g)** consistent with monocyte infiltration [358] **(Fig. 6h)** and an anti-inflammatory role for C1q [359]. Finally, we found that Dianeal-PD4 induced M(IL-4) in WT but not C1qa-deficient mice, as measured by the intracellular expression of RELMα, Ym1, and Arg (**Fig. 6i**) and protein secretion of RELMα and Ym1 into the peritoneal cavity (**Fig. 6j**). Notably, we found that Dianeal-PD4 also induced a moderate proliferation of pMϕs in WT but not C1qa-deficient mice (**Fig. 6k**).

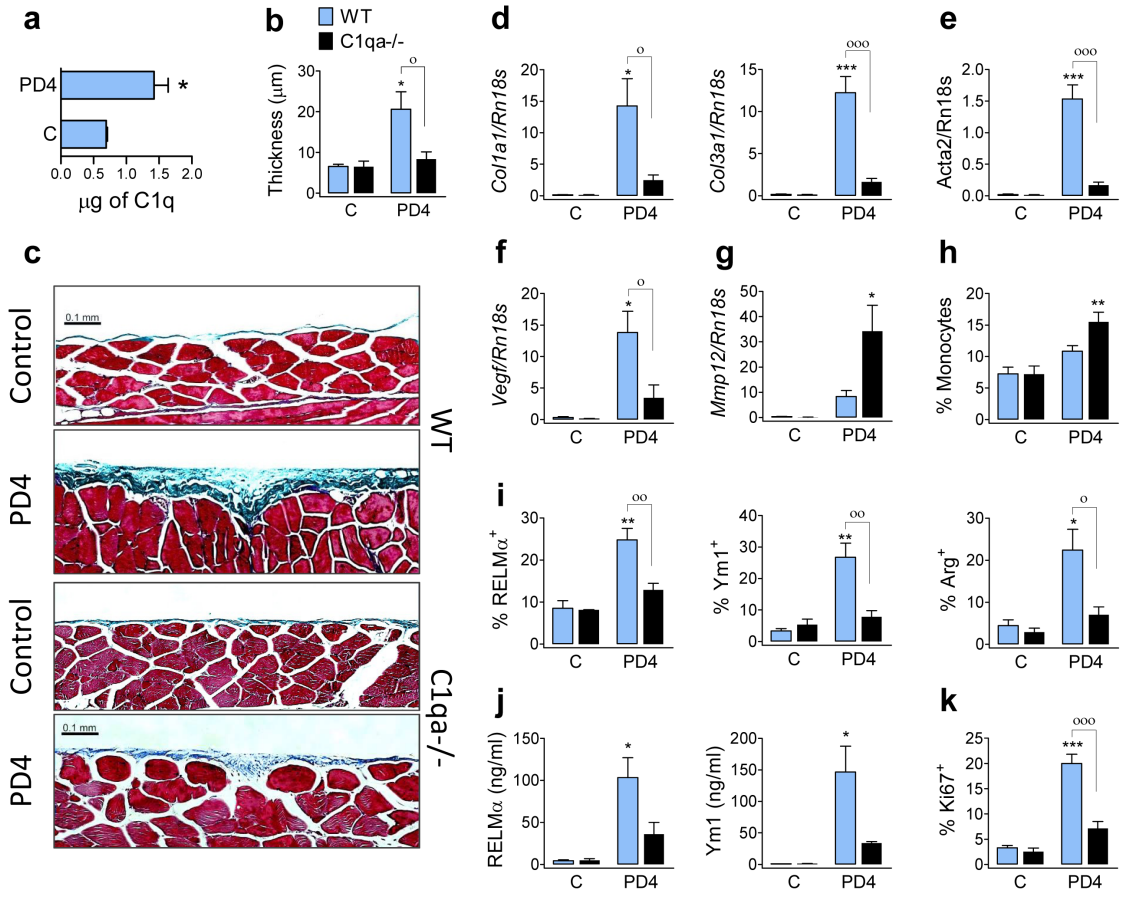


Figure 6. C1q-dependent enhancement of type 2 responses in a model of peritoneal fibrosis. WT or C1qa$^{-/-}$ mice were either untreated (C) or treated with a total of 14 deliveries of 500 µl of Dianeal PD-4 (i.p.) on alternate days. Samples were analyzed a day after the last delivery. **(a)** Absolute amount of C1q in the peritoneal washes as determined by ELISA. **(b)** Quantification of the thickness of the submesothelial compact zone from **(c)** microscopy of Masson's trichrome stained parietal peritoneum slices (scale bars, 0.1 mm). Amplification of **(d)** *Col1a1, Col3a1*, **(e)** *Acta2*, **(f)** *Vegf*, and **(g)** *Mmp12*-encoding mRNA in peritoneal tissue. **(h)** Percentage of infiltrating monocytes as quantified by FACS. **(i)** Expression of RELMα, Ym1, and Arg by peritoneal macrophages. **(j)** Analysis of secreted RELMα and Ym1 in the peritoneal space. **(k)** Expression of Ki67 by peritoneal macrophages. Results are representative from two independent experiments (means ± SEM) (untreated: 3 mice, PD4: 6 mice). ANOVA followed by the Bonferroni multiple-comparison test or Student's *t*-test (a) was used. $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$ when compared with control group; $^{\circ}p < 0.05$, $^{\circ\circ}p < 0.01$, and $^{\circ\circ\circ}p < 0.001$ when WT vs. C1qa$^{-/-}$ mice treated with Dianeal PD-4 are compared.

Discussion

Accumulating evidence suggests that local tissue factors and not necessarily origin determine the functional phenotype of macrophages [360]. We show here that ligands for the unconventional cell surface myosin18A act in a tissue-specific manner to enhance IL-4Rα-mediated macrophage proliferation and activation, with consequences for tissue repair, parasite killing, and fibrosis. Myo18A on alveolar macrophages recognizes SP-A but not C1q, while Myo18A on peritoneal macrophages recognizes C1q but not SP-A. All three of these factors are also positively regulated by IL-4 suggesting that in the tissues there is a local response that functions to dramatically amplify the type 2 effector response. SP-A and C1q both act via Myo18A but in a tissue-specific manner suggesting the involvement of local co-receptors that work in concert with Myo18A. C1q's lack of action on alveolar macrophages can be explained by the fact that alveolar macrophages do not express some C1q receptors [361]. Among the putative C1q receptors on peritoneal macrophages, gC1qR (also known as p33) lacks a trans-membrane domain, and signal transduction relies on partnerships with trans-membrane proteins [362]. Interestingly, gC1qR/P33 forms a signaling complex with DC-SIGN and integrin β1 [363], which is also a partner of Myo18A [364].

The need for tissue-specific co-receptors suggests that the origin and/or characteristics of the tissue where macrophages reside may condition their capacity to respond to IL-4. This is supported by our observation that alveolar but not peritoneal macrophages were able to proliferate in response to IL-4 *in vitro*. Besides having a different origin from peritoneal macrophages (fetal monocytes vs. primitive yolk sac [54]), alveolar macrophages have a unique phenotype that is determined by the alveolar environment, rich in surfactant components and high levels of oxygen [2]. Tissue-specific macrophage phenotypes are defined by unique transcription factors [365], and a recent analysis highlighted that alveolar macrophages of embryonic origin preferentially express the transcription factor Wwtr1 [56]. Notably, Wwtr1 is not expressed in peritoneal macrophages [366].

The observation that C1q can act locally to promote type 2 responses is consistent with described roles for C1q independent of the classical complement pathway [359, 367-370]. For example, C1q is produced by dermal microvascular endothelial cells in injured but not intact skin, and promotes repair [368]. Similarly, C1q produced by endothelial cells in the tumor microenvironment stimulates division of melanoma cells

[369]; in addition, C1q produced by decidual endothelial cells during pregnancy directs local cell migration and tissue remodeling [370]. The relevance of C1q as a tissue-specific factor is further supported by the fact that unlike most other complement components, which are produced in the liver, the majority of C1q is produced by myeloid cells in the peripheral tissues [371]. We show here that by enhancing M(IL-4) activation C1q plays a critical role in a clinically relevant model of dialysis-induced peritoneal fibrosis. This is consistent with human studies in which C1q is strongly associated with increased fibrosis of skeletal muscle [372].

The finding that SP-A promotes IL-4Rα-mediated activation and proliferation of alveolar macrophages defines an entirely new role for SP-A in the lung. In the context of a lung migrating helminth infection, SP-A acted as an important mediator of the host protective type 2 response to both control parasite numbers and repair lung damage. Enhancement of M(IL-4) may underlie the previously reported contribution of SP-A to tissue integrity in other models of acute lung injury [336, 373]. The pro-type 2 effects we report here contrast with reports that associate SP-A with protection in asthma [297]. However, in addition to promoting M(IL-4) and proliferation of macrophages, the already reported anti-inflammatory properties of SP-A [11, 336, 338, 339, 373] may suppress the strong inflammatory responses that are responsible for more severe asthma. Our data are supported by a recent study demonstrating that the closely related but far less abundant SP-D has IL-4 promoting effects similar to those of SP-A [374]. Because SP-D is critical for the turnover of surfactant [375, 376] there is a near absence of SP-A in SP-D-deficient mice [376]. The marked reduction of SP-A levels together with the accumulation of surfactant lipids [376] could contribute to observed effects in SP-D-deficient mice. In contrast, lung morphology, SP-D levels, and alveolar phospholipid pool sizes are unaltered in SP-A-deficient mice [377].

In conclusion, we have found that SP-A and C1q are tissue-specific factors that act through Myo18A to amplify local IL-4Rα-mediated macrophage activation. It is likely that other secreted soluble defense collagens present in distinct tissues are Myo18A ligands and will play similar roles. The common structural characteristic of these host defense proteins is their collagen domain, which mediated the IL-4Rα enhancing features of SP-A. SP-A, C1q, and Myo18 are highly conserved across mammalian species, and we have demonstrated the ability of SP-A and Myo18A to enhance human M(IL-4). Consequently, our findings have the potential to provide entirely new directions for

tissue-specific targeting of M(IL-4)-driven pathologies such as pathological fibrosis [131], a leading killer worldwide. Indeed manipulation of alveolar macrophages has been proposed as an attractive strategy for the treatment of pulmonary fibrosis [8]. The identification of the tissue-specific Myo18A co-receptors in the lung and other tissues will be the critical next step in developing strategies to regulate local macrophage activation and proliferation.

VIII. CHAPTER 3

Surfactant protein A enhances IL-4-mediated signaling via PKCζ and Akt in alveolar macrophages

Abstract

Interleukin (IL)-4 induced alternative activation and proliferation of macrophages involves intracellular activation of STAT6 and PI3K-Akt signaling. Surfactant Protein (SP)-A is a lung factor that enhances both IL-4-dependent activation as well as proliferation of alveolar macrophages via the unconventional Myosin18A. The mechanism by which SP-A and IL-4 synergistically enhance activation and proliferation of alveolar macrophages remains elusive. Here we show that SP-A, through engagement of the cell surface Myo18A receptor, enhanced PI3K activation in isolated rat alveolar macrophages. Furthermore, downstream of PI3K, PKCζ activation by SP-A selectively promoted the alternative activation of alveolar macrophages while Akt activation by SP-A selectively promoted IL-4-induced proliferation. These data show that SP-A is a lung-specific factor that enhances the amplitude of IL-4-induced signal in alveolar macrophages by activating PI3K-Akt and a complementary PKCζ-mediated signaling that feeds into STAT6 pathway.

Introduction

During type-2 immune responses, IL-4 and IL-13 signaling through the IL-4Rα triggers proliferation [5] and a specialized activation program of macrophages that has evolved as a specific immune response to expulse parasites [4]. Engagement of IL-4Rα ligands induces dimerization of this receptor with the common gamma chain Yc subunit forming a signaling complex. The IL-4R complex itself lacks intrinsic kinase activity, but the cytoplasmic tails of its subunits are each associated with the member of the JAK family JAK-1 [378]. IL-4R complex activates JAK-1, resulting in its auto and trans-phosphorylation as well as phosphorylation of target tyrosine residues in the cytoplasmic domains of both receptor chains. Phosphorylated tyrosine residues of the IL-4R complex act as docking sites for downstream adaptor and signaling molecules such as IRS-2, STAT6 and SHP-1 [100]. Of those signaling molecules, activated STAT6 is the master regulator of alternative macrophage activation [365]. However, it has also been shown that IL-4-induced proliferation of peritoneal macrophages is STAT6-dependent [304]. Alternatively, IRS-2 has been shown to serve as initiator of PI3K/Akt signaling pathway, which induces the proliferation and cell survival of macrophages [379]. Additionally, Ruckerl et al identified Akt as an important downstream target of PI3K signaling during IL-4-induced alternative activation [379].

Although type-2 immunity is a protective response to parasites, T_H2 responses are exacerbated during important non-communicable diseases of contemporary society. Consequently, alternatively activated macrophages are central effectors of type-2 immunity and play important roles in allergy, asthma, and lung fibrosis [131]. In the lung, IL-4Rα-activated macrophages express arginase, insulin-like growth factor 1 (IGF-1) and IL-10, all of which contribute to rapid resolution of tissue damage induced by the lung-migrating parasite *Nippostrongylus brasiliensis* [6]. Other studies have confirmed the role of alternatively activated alveolar macrophages during lung repair. For instance, it has been shown that alternatively activated lung macrophages control severe respiratory syncytial virus-induced lung injury [380]. Finally, it has been observed that alveolar macrophages are critical promoters of both the local induction and maintenance of type 2-dependent lung fibrosis [131].

Alveolar macrophages together with the respiratory epithelium are covered by pulmonary surfactant, a lipid-protein network that contains associated proteins essential for keeping the alveolus open and for host defense [9, 11]. The two principal surfactant components involved in innate immunity in the alveolus are surfactant proteins A (SP-A) and D (SP-D) [11]. SP-A is an oligomeric extracellular protein that is found mainly in the alveolar fluid, associated with surfactant extracellular membranes that line the alveolar epithelium and with alveolar cells. SP-A recognizes pathogen-associated molecular patterns on some microorganisms, resulting in aggregation, opsonization, or permeabilization of microorganisms and facilitation of microbial clearance [11, 250]. Moreover, SP-A is also able to bind to membrane receptors present in macrophages, epithelial cells, and lymphocytes, modifying their response to different stimuli [11]. SP-A blocks the binding of TLR ligands to their receptors by direct SP-A interaction with TLR4 [273], TLR2 [276], the TLR co-receptor MD2 [276], and CD14 [16, 17]. Furthermore, SP-A modifies macrophage response to TLR ligands by modulating signaling cascades. For example, SP-A increases the expression of negative regulators of TLR-signaling, such as IRAK-M (14) and β-arrestin 2 (13), thereby inhibiting LPS-induced stimulation of macrophages. Moreover, SP-A promotes PKCζ activation and IκBα stabilization through mechanisms that require SP-A endocytosis by macrophages [280]. Internalized SP-A also inhibits IκBα, ERK, p38, and Akt phosphorylation by macrophages stimulated with TLR2 and TLR-4 ligands [281]. Although the role of SP-A and SP-D during type 2 immunity remains less explored, a recent publication showed that SP-D induction is essential for immunity to helminth infection, an observation that correlated with elevated expression of markers of alternative activation by alveolar macrophages in response to SP-D [374]. Similarly, we have shown that SP-A is a lung factor that acts through Myo18A to enhance IL-4Rα-mediated macrophage activation and proliferation with consequences for lung repair and parasite control during *Nippostrongylus brasiliensis* infection [381]. However, how SP-A promotes IL-4-induced activation and proliferation of alveolar macrophages remains unknown.

Here we show that SP-A, via the unconventional myosin Myo18A, enhanced PI3K activation and the phosphorylation of its down-stream targets PKCζ and Akt. Accordingly, the activation of Akt by SP-A enhanced IL-4-induced proliferation, while

the activation of PKCζ by SP-A increased IL-4 induced alternative activation of alveolar macrophages. Thus, our data suggest that SP-A activates a signaling pathway that feeds into the signaling initiated by IL-4 enhancing the amplitude of its signal.

Materials and Methods

Materials

Cell culture media and reagents were acquired from Lonza (Basel, Switzerland). Western blotting materials were obtained from Bio-Rad (Hercules, California). All other reagents were of analytical grade, purchased from Sigma (St. Louis, Missouri) unless otherwise specified.

Isolation, purification and characterization of native SP-A

Surfactant protein A was isolated from BAL of patients with alveolar proteinosis using a sequential butanol and octylglucoside extraction [14, 173, 177, 339, 340]. The purity of SP-A was checked by one-dimensional SDS-PAGE in 12 % acrylamide under reducing conditions and mass spectrometry. The oligomerization state of SP-A was assessed by electrophoresis under non-denaturing conditions [173, 177], electron microscopy [173], and analytical ultracentrifugation as reported elsewhere [177]. SP-A consisted of supratrimeric oligomers of at least 18 subunits. Each subunit had a relative molecular mass (Mr) 36 kDa. The endotoxin content of native or recombinant human SP-A was < 0.1 endotoxin units /mg of SP-A as determined by Limulus amebocyte lysate assay (GenScript, Piscataway, New Jersey).

Animal handling

Rat alveolar macrophages were obtained from Sprague–Dawley male rats. Rats (~350 g) were anesthetized with ketamine (80 mg/kg; Merial, Duluth, GA) and xylazine (10 mg/kg; Bayer, Leverkusen, Germany) and the cardiopulmonary block was extracted to perform bronchoalveolar lavages with PBS (0.2 mM EDTA). All animals received humane care in accordance with the Guide for the Care and Use of Laboratory Animals [312] and Spanish guidelines for experimental animals.

Isolation and culture of primary aMϕ´s

Bronchoalveolar cells were separated from lavage fluid by centrifugation (250 g, 7 min). The sedimented cells were washed twice with PBS, and the cell pellet was resuspended in RPMI 1640 medium (10% heat-inactivated FBS, 100 U/ml penicillin, and 100 mg/ml streptomycin, supplemented with 2 mM glutamine) (Lonza). Alveolar macrophages were purified by adherence for 90 min at 37°C under a 95% air-5%

CO_2 atmosphere in 150-cm^2 culture flasks as reported previously [313]. Adherent cells were 94.0 6 1.1% viable (trypan blue exclusion test). To evaluate the purity of the isolated macrophages, rat alveolar macrophages immune-stained with anti-CD11c (Serotec, Kidlington, U.K.) were analyzed by flow cytometry. Adherent cells were found to be composed of 90 ± 1 % alveolar macrophages.

Incubation conditions

Adherent cells were gently scraped, plated in 96-well plastic dishes (7.5 x 10^4 cells/well) in 0.2 ml RPMI 1640 medium with 5% FBS, and pre-cultured overnight. Cells were cultured in the presence or absence of IL-4 (1 µg/mL) (ImmunoTools, Berlin, Germany) and/or SP-A (25, 50 and 100 µg / mL). Lower doses of IL-4 (0.1-1 µg/mL) were also assayed; however, alternative activation and proliferation of alveolar macrophages was significantly induced only with concentrations of IL-4 above 1 µg/mL. Different concentrations of inhibitors were titrated to minimize innocuous effects and toxicity. After titration the following concentrations were used: 50 nM LY294002 (PI3K inhibitor) (Cell Signaling, Danvers, Massachusetts), 25 nM Akt Inhibitor VIII (Isozyme-Selective, Akti-1/2), 30 µM PKCζ Pseudosubstrate Inhibitor, Myristoylated (Calbiochem, Darmstadt, Germany). Cell viability was 97% under assay conditions. Macrophage cultures were plated in triplicate wells, and each series of experiments was repeated at least three times.

Arginase activity assay

Arginase activity was measured as previously reported [349]. Briefly, rat alveolar macrophages were lysed with 50 µl of 50 mM Tris–HCl pH 7.5, Triton X-100 0.1 %, 1 mM benzamidine, 200 µg/mL aprotinin, and 200 µg/ml leupeptin. After 30 min shaking at 4ºC, arginase was activated with 50 µL of 10 mM $MnCl_2$ and 50 mM Tris-HCl, pH 7.5, for 10 min at 55º C. L-arginine hydrolysis was measured by incubating the cell lysate with 25 µL of 0.5 M L-arginine (Sigma) (pH 9.7) at 37ºC for 1 h. The reaction was stopped by addition of 200 µL H_2SO_4 /H_3PO_4 /H_2O (1:3:7 v/v). The produced urea was quantified at 570 nm after addition of 25 µL of α-isonitrosopropiophenone (dissolved in 100% ethanol) followed by heating at 99ºC for 45 min. Urea production was normalized to cell number for each treatment by quantifying cells with the WST-1 reagent (Roche), following manufacturer' instructions. One unit of arginase activity is defined as the amount of enzyme that

catalyses the formation of 1 μmol urea per min.

Immunoprecipitation of PKCζ

Alveolar macrophages were stimulated with SP-A, IL-4 or combinations thereof for 8 minutes. After culture, cells were lysed at 4ºC for 30 min in 500 μL of lysis buffer: 50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 0.5 mM EDTA, IGEPAL 0.5%, 1 mM benzamidine, 200 μg/mL aprotinin, 200 μg/mL leupeptin, 1 mM phenylmethylsulfonyl fluoride (PMSF), 20 mM β-glycerophosphate, 10 mM NaF, 10 mM sodium pyrophosphate and 2 mM orthovanadate. The lysates were centrifuged at 10 000 × g for 10 min, and the supernatants were precleared by adding protein A-agarose (50 μL) and incubated at 4°C for 45 min, followed by centrifugation at 10 000 × g for 10 min. The precleared supernatant was incubated with anti-PKCζ antibody (Cell Signaling, Danvers, Massachusetts) or control IgG at 4°C overnight, after which 50 μL of protein A-agarose (Santa Cruz Biotechnology, Dallas, Texas) was added for 3 h at 4°C with gentle rotation. The immune complexes were collected by centrifugation at 10 000 × g for 5 min at 4°C, washed three times with cold lysis buffer, and released by boiling with 5× Laemmli loading buffer. Phosphorylation of PKCζ was subsequently analyzed by Western blot using an anti-Phospho-PKCζ/λ (Thr410/403) and anti-PKCζ (Cell Signaling) as described below.

Western blot analysis

Alveolar macrophages were stimulated with SP-A, IL-4 or combinations thereof for 90 minutes to determine STAT6 phosphorylation and 30 minutes for Akt phosphorylation. Cells were lysed by shaking 30 min at 4ºC with a buffer containing: 10 mM HEPES, pH 7.9, 15 mM MgCl, 10 mM KCl, 0.5 mM EDTA, Triton X-100 0.2%, 1 mM benzamidine, 200 μg/mL aprotinin, 200 μg/mL leupeptin, 1 mM phenylmethylsulfonyl fluoride (PMSF), 20 mM β-glycerophosphate, 10 mM NaF, 10 mM sodium pyrophosphate and 2 mM orthovanadate. Samples were resolved by 10% (m/v) SDS-PAGE in reducing conditions and transferred to polyvinylidene fluoride membranes (PVDF). After blocking with 2.5% (m/v) skim milk, membranes were washed and incubated with anti- phospho STAT6 (Tyr641) (Pierce Antibodies), phospho Akt (Ser473), STAT6, Akt and α-Tubulin (Cell Signaling) overnight at 4°C. The membranes were washed and incubated with horseradish-peroxidase-labeled anti-rabbit/mouse IgG for 30 minutes at room temperature (RT), and exposed to ECL

reagents. Immuno-reactive bands density was measured with the software Quantity One.

Cell proliferation assays

Detection of BrdU incorporation was performed by ELISA according to the manufacturer's instructions (Cell Signaling). The retention of the cells at the bottom of the well after the removal of the supernatant was checked by the wst-1 assay (Roche). Cell number in all treatments was similar before and after supernatant removal.

siRNA-targeted silencing of Myo18A

After isolation, primary alveolar macrophages were resuspended in Amaxa® mouse macrophage nucleofector solution (Lonza) and nucleofected with 100 nM siRNA using a nucleofector 2b device (Lonza). Experiments were conducted using two Stealth siRNAs directed against rat Myo18A (RSS322720 and RSS322721) (Applied Biosystems, Carlsbad, California). Medium GC Stealth siRNA was used as control (12935300) (Applied Biosystems). Myo18A expression was detected by Western blot analysis with an anti-Myo18A antibody as reported previously [381]. After 48 hours post nucleofection, Myo18A expression was reduced 72 ± 4% for RSS322720 and 71 ± 5% for RSS322721 compared to control. At this time-point, cells were stimulated.

Statistics

Statistical evaluation of different groups was performed by analysis of variance (ANOVA) followed by the Bonferroni multiple comparison test. An α level ≤ 5% ($p \leq 0.05$) was considered significant. All statistical calculations were performed using PRISM, (Graphpad La Jolla, CA).

Results

SP-A enhances IL-4-mediated activation and proliferation of alveolar macrophages

We have previously shown that SP-A enhances IL-4Rα-mediated activation and proliferation of alveolar macrophages in mice, rats and humans [381]. IL-4Rα-dependent activation of STAT6 regulates arginase (Arg1) and Mannose receptor (CD206) expression by macrophages [365]. Furthermore, it has also been shown that IL-4Rα signalling promotes proliferation of macrophages [5]. To confirm the effect of SP-A on IL-4Rα-activated alveolar macrophages we measured arginase activity, CD206 expression and proliferation of rat alveolar macrophages cultured in the presence of IL-4, SP-A and combinations thereof.

As expected, IL-4 induced arginase activity, CD206 expression and the proliferation of alveolar macrophages (Fig. 1a). Furthermore, we confirmed that SP-A dose-dependently enhanced IL-4-mediated effects on alveolar macrophages as measured by the induction of arginase cativity, CD206 expression and BrdU incorporation to the newly synthetized DNA of proliferating macrophages (Fig. 1a). In our previous report we identified the unconventional myosin, Myo18A (aka SP-R210) as the receptor for SP-A involved in the enhancement of IL-4 effects in alveolar macrophages [381]. To corroborate these observations, we silenced Myo18A gene using Myo18A-targeted siRNA and measured proliferation and arginase activity of IL-4 and/or SP-A treated alveolar macrophages. Figure 1b shows that silencing of Myo18A completely abrogated SP-A effects of IL-4-treated alveolar macrophages confirming our previous observations.

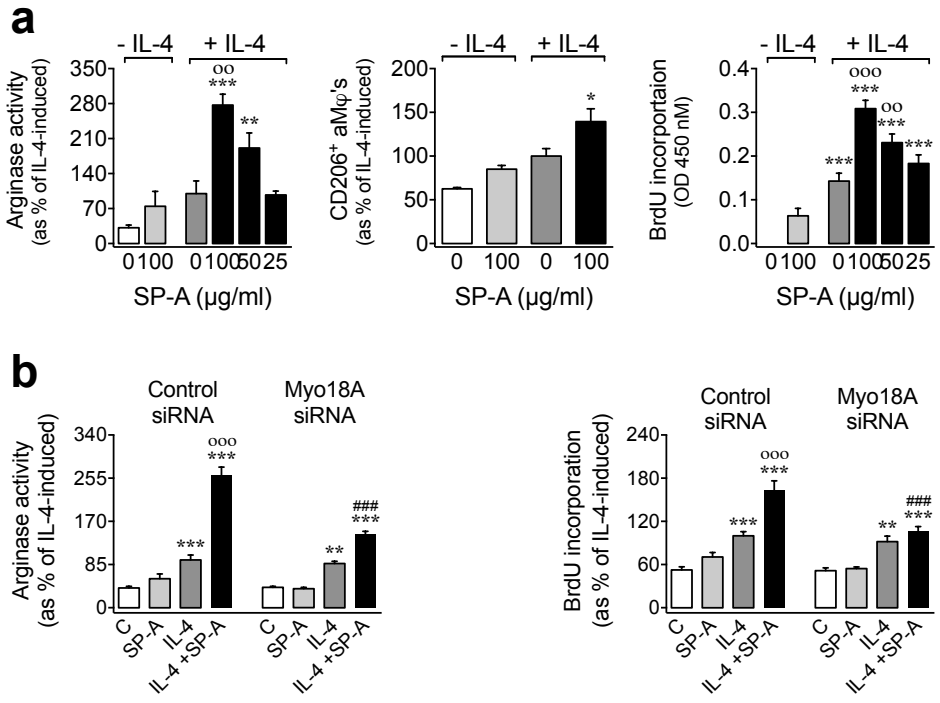


Figure 1. SP-A enhances IL-4-dependent activation and proliferation of alveolar macrophages. Purified rat aMϕ were left untreated or nucleofected with Myo18A or siRNA control. Subsequently, aMϕ were stimulated with or without IL-4 (1 µg/mL) in the presence or absence of SP-A (25, 50 and 100 µg/mL) and exposed to 10 µM BrdU for proliferation analysis. We measured ***(a)*** arginase activity, CD206 expression and BrdU incorporation to the newly synthesised DNA of alveolar macrophages that were not nucleofected. Alternatively, we measured ***(b)*** arginase activity and BrdU incorporation to the newly synthesised DNA of alveolar macrophages that were nucleofected with Myo18A or siRNA control. The results are presented as means (± SEM) from three different cell cultures with at least three biological replicates. ANOVA followed by the Bonferroni multiple-comparison test was used. *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$, when compared with untreated macrophages; $^{oo}p < 0.01$, and $^{ooo}p < 0.001$, when SP-A+IL-4-treated macrophages were compared with IL-4-treated macrophages; $^{###}p < 0.001$, when the effect of Myo18A siRNA is compared with siRNA control.

SP-A enhances IL-4 signalling through Myo18A receptor

Mechanistically, SP-A could act extracellularly enhancing IL-4 effects in macrophages by binding to IL-4 or IL-4Rα. However, silencing of Myo18A completely abrogated the effects of SP-A on IL-4-treated alveolar macrophages, suggesting that the synergy between SP-A and IL-4 must occur downstream from their respective receptors. Besides the well-established role of STAT6 during IL-4Rα signalling, it has

been shown that PI3K-Akt activation is essential for IL-4-driven murine macrophage activation and proliferation [379]. Therefore, to determine whether SP-A-Myo18A could induce a similar signalling to IL-4, we analysed STAT6 and Akt phosphorylation by macrophages cultured in the presence and absence of IL-4, SP-A and combinations thereof. As expected, IL-4 induced the phosphorylation of STAT6 and Akt by alveolar macrophages (Fig. 2a). Moreover, SP-A dose-dependently enhanced IL-4-induced STAT6 and Akt phosphorylation mediated by alveolar macrophages (Fig. 2a). Of note, SP-A and IL-4 together induced an additive phosphorylation of Akt; however SP-A alone was capable to induced Akt phosphorylation (Fig. 2a) supporting the idea that the effect of SP-A on IL-4-treated macrophages occurs at the signalling level. To show that Myo18A mediates SP-A signaling on alveolar macrophages, we silenced *Myo18A* and repeated STAT6 and Akt phosphorylation determinations in IL-4 and SP-A treated macrophages. We found that silencing of Myo18A abrogated the effects of SP-A on IL-4-induced STAT6 and Akt phosphorylation by alveolar macrophages (Fig. 2b). Notably, we observed that the silencing of Myo18A on alveolar macrophages also impaired the capability of SP-A to induce Akt phosphorylation by its own (Fig. 2b)

Myo18A is an unconventional myosin that does not operate as a traditional molecular motor in cells, having both intracellular and cell-surface locations [352]. Immune activation results in Myo18A localization on the cell surface, where it binds to SP-A with high affinity and thus is also known as SP-R210 [291, 338]. We have previously shown that blockade or silencing of Myo18A has similar effects on the SP-A enhancement of IL-4Rα-mediated macrophage activation and proliferation [381]. Similarly, blockade of Myo18A abolished the SP-A-mediated activation of Akt (Data not shown).

Therefore, these data suggests that SP-A-Myo18A signaling directly activates PI3K-Akt signaling by alveolar macrophages and synergises with IL-4 signalling to increase the activation and proliferation of alveolar macrophages.

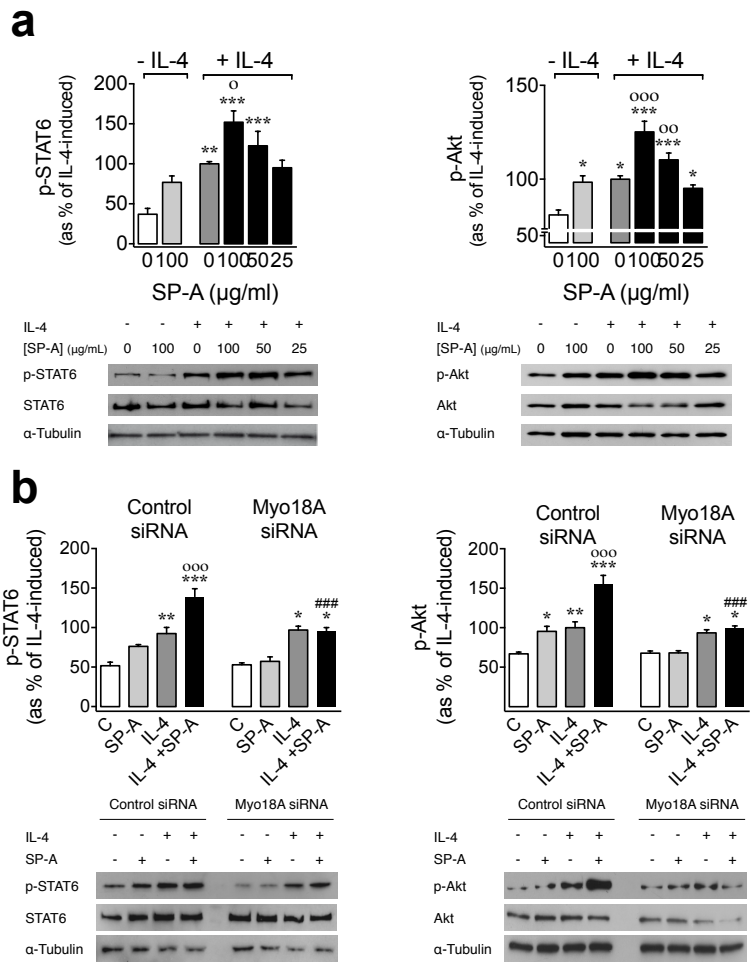


Figure 2. SP-A amplifies IL-4 signaling through Myo18A on alveolar macrophages. Purified rat aMϕ were left untreated or nucleofected with Myo18A or siRNA control. Subsequently, aMϕ were stimulated with or without IL-4 (1 μg/mL) in the presence or absence of SP-A (25, 50 and 100 μg/mL) and exposed to 10 μM BrdU for proliferation analysis. We measured ***(a)*** STAT6 and Akt phosphorylation by IL-4 and/or SP-A-treated alveolar macrophages that were not nucleofected. Alternatively, we measured ***(b)*** STAT6 and Akt phosphorylation by IL-4 and/or SP-A-treated alveolar macrophages that were nucleofected with Myo18A or siRNA control. A representative WB for p-STAT6 and p-Akt is shown in figures a and b. The results are presented as means (± SEM) from three different cell cultures with at least three biological replicates. ANOVA followed by the Bonferroni multiple-comparison test was used. *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$, when compared with untreated macrophages; $^{o}p < 0.05$, $^{oo}p < 0.01$, $^{ooo}p < 0.001$, when SP-A+IL-4-treated macrophages were compared with IL-4-treated macrophages; $^{###}p < 0.001$, when the effect of Myo18A siRNA is compared with siRNA control.

PI3K inhibitor blocks SP-A effects on IL-4 signaling

As mentioned before, a previous report showed that signaling via PI3K-Akt is essential for full IL-4-driven activation and proliferation of macrophages [379]. Alternatively, PKCζ another target of PI3K signaling [382], is critical for the IL-4-induced nuclear translocation and tyrosine phosphorylation of STAT6 and Jak1 activation allowing for Th2 differentiation [383-385]. Regarding SP-A signaling, it has been reported that SP-A activates a PI3K signal transduction pathway that up-regulates mannose receptor (CD206) by human macrophages [13]. Furthermore, previous publications have shown that SP-A induces PKCζ activation dampening the inflammatory macrophage activation triggered by LPS [310, 386, 387]. Therefore, we rationalized that the induction of PI3K signaling by SP-A could explain the enhancement of IL-4Rα-mediated macrophage proliferation and activation. To address this hypothesis, we inhibited PI3K activation (50nM LY294002) in IL-4 and SP-A-treated macrophages and analyzed the phosphorylation of its downstream targets Akt and PKCζ. Additionally, we assed the effect of PI3K inhibition on STAT6 phosphorylation, arginase activity and BrdU incorporation by alveolar macrophages cultured in the presence of IL-4, SP-A and combinations thereof. We observed that the addition of 50nM of the PI3K inhibitor LY294002 successfully inhibited PI3K activity as the presence of the inhibitor abolished the capability of SP-A and IL-4 to induce the phosphorylation of Akt and PKCζ in alveolar macrophages (Fig 3a-b). Moreover, the inhibition of PI3K abrogated SP-A enhancement of IL-4-induced STAT6 phosphorylation and arginase activity by alveolar macrophages (Fig. 3c-d). Critically, our results suggested that IL-4 does not depend on PI3K to induce STAT6 phosphorylation and the alternative activation of alveolar macrophages (Fig. 3c). In contrast, we observed that in the presence of PI3K inhibitor the proliferation of alveolar macrophages was completely impaired in all the treatments (Fig. 3e).

These results show that PI3K signaling directly instructs macrophage proliferation and suggest that there is a crosstalk between STAT6 and PI3K that combine IL-4 and SP-A signaling to enhance alternative activation of alveolar macrophages potentially mediated by Akt or PKCζ.

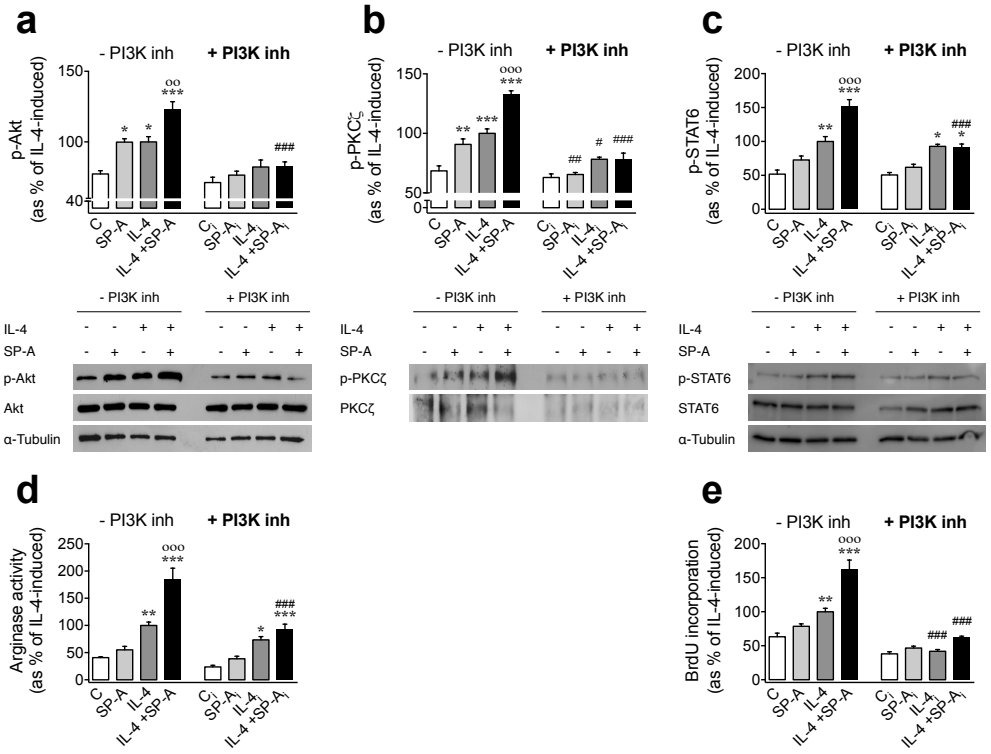


Figure 3. PI3K inhibitor abrogates SP-A effects on IL-4 signaling by alveolar macrophages. Purified rat aMϕ were pre-treated with 50 nM LY294002 (PI3K inhibitor) or vehicle (DMSO) for two hours. Subsequently, cells were stimulated with or without IL-4 (1 µg/mL) in the presence or absence of SP-A (100 µg/mL) and exposed to 10 µM BrdU for proliferation analysis. We measured ***(a)*** Akt, ***(b)*** PKCζ and ***(c)*** STAT6 phosphorylation; ***(d)*** arginase activity and ***(e)*** BrdU incorporation to the newly synthesised DNA of IL-4 and/or SP-A-treated alveolar macrophages that were cultured with or without PI3K inhibitor. A representative WB for p-Akt, p- PKCζ and p-STAT6 is shown. The results are presented as means (± SEM) from three different cell cultures with at least three biological replicates. ANOVA followed by the Bonferroni multiple-comparison test was used. *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$, when compared with untreated macrophages; $^{oo}p < 0.01$, and $^{ooo}p < 0.001$, when SP-A+IL-4-treated macrophages were compared with IL-4-treated macrophages; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, and $^{\#\#\#}p < 0.001$, when the effect of LY294002 is compared with vehicle.

Akt activation controls SP-A and IL-4-mediated macrophage proliferation

One of the best-characterized targets of activated PI3K is the AKT/mTOR signaling pathway [388]. To test whether Akt mediates the crosstalk between STAT6 and PI3K signaling during IL-4 and SP-A stimulation of alveolar macrophages, we inhibited Akt activation with the Akt Inhibitor VIII (25 nM). We observed that the inhibition of Akt did not affect STAT6 phosphorylation and arginase activity by alveolar macrophages in any of the treatments (Fig. 4a). In contrast, Akt Inhibitor VIII

dramatically impaired proliferation of alveolar macrophages induced by IL-4 alone or in combination with SP-A (Fig. 4b) confirming previous observations [379].

These results demonstrate that whereas Akt activation is essential for macrophage proliferation, activated Akt is not directly involved in the signaling pathway that promotes alternative activation of alveolar macrophages.

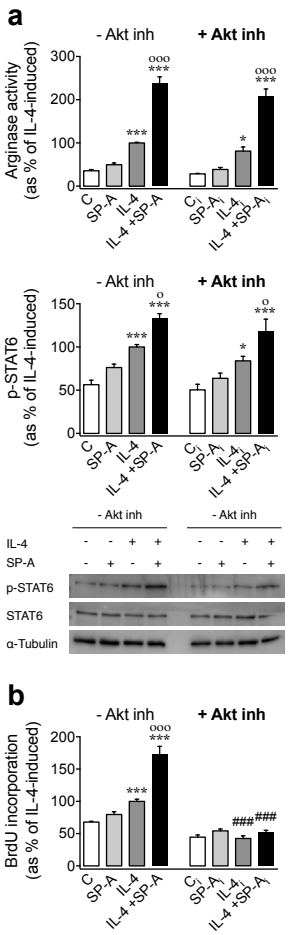


Figure 4. Akt activation controls SP-A and IL-4-mediated macrophage proliferation. Purified rat aMϕ were pre-treated with 25 nM Akt Inhibitor VIII or vehicle for two hours. Subsequently, cells were stimulated with or without IL-4 (1 μg/mL) in the presence or absence of SP-A (100 μg/mL) and exposed to 10 μM BrdU for proliferation analysis. We measured ***(a)*** arginase activity, STAT6 phosphorylation and ***b)*** BrdU incorporation to the newly synthesised DNA of IL-4 and/or SP-A-treated alveolar macrophages that were cultured with or without Akt inhibitor. A representative WB for p-STAT6 is shown. The results are presented as means (± SEM) from three different cell cultures with at least three biological replicates. ANOVA followed by the Bonferroni multiple-comparison test was used. *$p < 0.05$ and ***$p < 0.001$, when compared with untreated macrophages; $^{o}p < 0.05$ and $^{ooo}p < 0.001$, when SP-A+IL-4-treated macrophages were compared with IL-4-treated macrophages; $^{###}p < 0.001$, when the effect of Akt Inhibitor VIII is compared with vehicle.

PKCζ activation mediates SP-A enhancement of IL-4-induced activation of alveolar macrophages

As mentioned previously, another target of the PI3K signaling pathway activated either by IL-4 or SP-A is PKCζ [310, 383, 384, 386, 387]. To determine whether the effect of SP-A on the IL-4-induced alternative activation of alveolar macrophages is mediated by PKCζ, we inhibited PKCζ with a PKCζ

Pseudosubstrate. We observed that in the presence of PKCζ inhibitor (30 μM), SP-A was unable to enhance IL-4-induced arginase activity by alveolar macrophages (Fig. 5a). Accordingly, PKCζ inhibition prevented SP-A's enhancement of IL-4-induced STAT6 phosphorylation by alveolar macrophages (Fig. 5a). Of note, the effect of IL-4 on STAT6 phosphorylation and arginase activity by alveolar macrophages was not affected by the presence of PKCζ inhibitor (Fig. 5a). Similarly, the same concentration of PKCζ inhibitor did not affect alveolar macrophages proliferation in any treatment (Fig. 5b).

These results demonstrate that SP-A enhances IL-4-induced alternative activation of alveolar macrophages by sustaining the PI3K-PKCζ-STAT6 signaling loop initiated by IL-4. Furthermore, our data establishes that PKCζ signaling intersects with activated STAT6 to achieve an effective activation without directly affecting proliferation of alveolar macrophages.

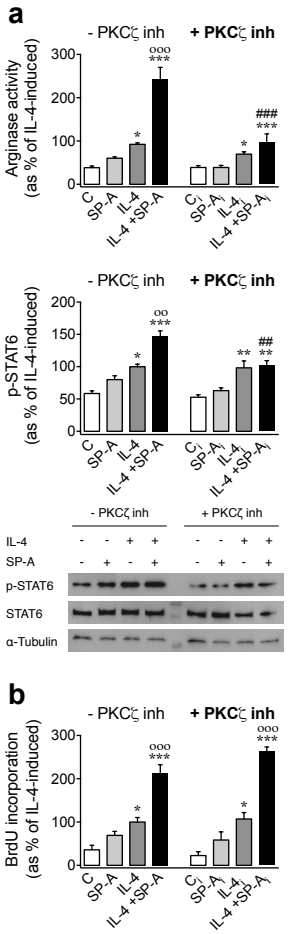


Figure 5. SP-A enhancement of IL-4-mediated activation of alveolar macrophages requires PKCζ activation. Purified rat aMϕ were pre-treated with 30 μM of PKCζ Pseudosubstrate Inhibitor or vehicle for two hours. Subsequently, cells were stimulated with or without IL-4 (1 μg/mL) in the presence or absence of SP-A (100 μg/mL) and exposed to 10 μM BrdU for proliferation analysis. We measured ***(a)*** arginase activity, STAT6 phosphorylation and ***(b)*** BrdU incorporation to the newly synthesised DNA of IL-4 and/or SP-A-treated alveolar macrophages that were cultured with or without PKCζ Pseudosubstrate. A representative WB for p-STAT6 is shown. The results are presented as means (± SEM) from three different cell cultures with at least three biological replicates. ANOVA followed by the Bonferroni multiple-comparison test was used. *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$, when compared with untreated macrophages; $^{oo}p < 0.01$, and $^{ooo}p < 0.001$, when SP-A+IL-4-treated macrophages were compared with IL-4-treated macrophages; $^{\#\#}p < 0.01$, and $^{\#\#\#}p < 0.001$, when the effect of PKCζ Pseudosubstrate is compared with vehicle.

Discussion

Accumulating evidences suggest that local factors in the lung largely determine the phenotype of alveolar macrophages [57, 58], which help to maintain tissue homeostasis and act as sentinels of injury. Following injury, IL-4-activated macrophages make a substantial contribution to wound healing [127]. In this context, we had shown [381] that SP-A is a local factor that acts to enhance IL-4-dependent proliferation and activation of alveolar macrophages, resulting in better lung injury resolution following infection with the lung-migrating nematode *Nippostrongylus brasiliensis*. IL-4-induced alternative activation and proliferation of macrophages requires intracellular activation of STAT6 and PI3K-Akt signaling. Here we show that binding of SP-A to the cell surface receptor Myo18A induced the activation of PI3K, which in turn activated its downstream targets Akt and PKCζ. SP-A-mediated Akt and PKCz activation induced differential effects on IL-4-activated alveolar macrophages: whereas SP-A induction of the PI3K-Akt signaling axis increased IL-4-induced proliferation, SP-A-induced PI3K-PKCζ signaling axis increased IL-4-induced alternative activation by amplifying STAT6 signaling in alveolar macrophages.

It has been shown that SP-A-mediated PI3K activation enhances the expression of CD206, a widely used marker of alternative activation of macrophages [13]. Furthermore, our previous report [381] identified the unconventional myosin, Myo18A (aka SP-R210) as the receptor for SP-A involved in the enhancement of IL-4Rα-mediated macrophage activation and proliferation. The present study confirms these observations by showing that the silencing of Myo18A impairs SP-A-mediated PI3K activity and as consequence SP-A-mediated enhancement of IL-4-induced activation and proliferation of alveolar macrophages. Although it has been reported that immune activation results in Myo18A localization on the cell surface of macrophages [291, 338, 381], the mechanism by which this receptor, that lacks a trans-membrane domain, transduces extracellular signals to the cytosol is not known. A recent study provided insights of the potential mechanism by which Myo18A could mediate SP-A-induced PI3K activation. This study showed that Myosin 18A co-immunoprecipitates with SHP-2 [389], a tyrosine phosphatase that is required for mediating PI3K-Akt activation [390], which is also one of the molecules that is recruited upon activation of the IL-4R complex [100]. Furthermore, Myo18A has been

shown to partner with integrin β1 [364] an integrin that activates PI3K signalling [391] and which cross-linking induces the assembly of a SHP-2/Gab2 complex in hematopoietic cells [392]. Therefore, Myo18A signalling could potentially rely on partnership with the trans-membrane protein integrin β1 to recognise and transduce the signal upon SP-A ligation.

By using Triciribine, a nucleoside that selectively inhibits the cellular phosphorylation/activation of Akt1, Akt2 and Akt3 [393], a previous *in vivo* study showed that the IL-4-driven macrophage proliferation is PI3K-Akt dependent [379]. In contrast to Triciribine, Akt Inhibitor VIII at the concentrations used in the present study, is a selective inhibitor of Akt1 [394]. Therefore, our data shows that selective inhibition of Akt1 is enough to prevent IL-4-induced macrophage proliferation, suggesting a critical role of Akt1 during this process. Furthermore, our study shows that SP-A and IL-4 induce the activation of PI3K-Akt in a synergistic manner resulting in an increased proliferation of alveolar macrophages. Critically, SP-A-mediated enhancement of the IL-4-induced proliferation of macrophahes was also abrogated by selective inhibition of Akt1, suggesting that the activation of PI3K-Akt by SP-A and IL-4 is convergent. The studies of Ruckerl et al [379] also showed that Triciribine impairs IL-4-mediated alternative activation of macrophages. Our results showed that low concentrations of Akt inhibitor VIII do not impair IL-4 and SP-A-induced alternative activation of alveolar macrophages, suggesting that Akt1 is dispensable for this process. However, higher concentrations of Akt Inhibitor VIII, which are supposed to inhibit Akt1 but also Akt2 and Akt3 [394], impaired IL-4 and SP-A-induced proliferation and alternative activation of alveolar macrophages (data not shown) confirming Ruckerl et al [379] observations.

All together these observations sugest that there is a crosstalk between PI3K and STAT6 signaling that regulates alternative activation of macrophages. Our study also suggested that SP-A is capable to amplify this crosstalk resulting in an enhanced alternative activation of alveolar macrophages. By activating a PI3K-PKCζ signaling axis SP-A enhanced IL-4-induced STAT6 signaling by alveolar macrophages. Previous publications have shown that PKCζ is required for the nuclear translocation and tyrosine phosphorylation of STAT6 and Jak1 activation during $CD4^+$ T cell differentiation in response to IL-4 [384, 385]. Whereas IL-4-

induced $CD4^+$ T cell differentiation into T_H2 cells apeared to be absolutey dependent on PKCζ [384, 385], our study shows that in alveolar macrophages IL-4-induced alternative activation of macrophages occurs independently of PKCζ signaling. However, PKCζ signaling is activated by SP-A to enhance IL-4-induced STAT6 activation. Previous publications have shown that SP-A promotes PKCζ activation and IκBα stabilization inducing dampening of the inflammatory macrophage activation triggered by LPS [310, 386, 387]. Here we define a novel function of SP-A-activated PKCζ during the IL-4-induced alternative activation of macrophages. Further studies are required to reveal more details of the mechanism by which SP-A mediates PKCζ activation; a better understanding of this process might be important to understand why the inhibition of the PI3K-PKCζ signaling axis in alveolar macrophages abrogates the effects of SP-A without affecting the effect of IL-4.

This study reveals a so far neglected layer of a tissue-specific pathway in the regulation of macrophage function in the context of type 2 immunity. Although it is known that IL-4 reduces the expression of IL-4Rα on the cell surface of alveolar macrophages [381], we have shown before that IL-4 positively regulates the expression of SP-A and its receptor Myo18A [381]. This data suggest that the type-2 effector function of macrophages will depend on the additive effects of different factors present in the local response. In summary, we show that SP-A enhances IL-4-mediated macrophage activation and proliferation by inducing a signaling pathway that converges into IL-4 signaling amplifying and sustaining IL-4 signals. By activating PI3K-Akt signaling, SP-A synergizes with IL-4 to expand the population of alveolar macrophages. Furthermore, we show that besides the induction of STAT6 phosphorylation by IL-4 there are other complementary pathways that can be triggered by SP-A to amplify the alternative activation of macrophages. In particular, we have shown that by activating PKCζ, SP-A establishes a crosstalk between PIK3 and STAT6 signaling that increased IL-4-dependent STAT6 activity and therefore the alternative activation of macrophages.

IX. CONCLUSIONS

The research presented in this doctoral dissertation provides new evidences about the immune properties of surfactant protein A (SP-A) and reveals further details about the molecular mechanisms by which SP-A modulates classical and alternative activation of alveolar macrophages. Taking all the results into consideration, we conclude that:

- SP-A limits classical activation of macrophages induced by either IFN-γ plus LPS or both factors separately. The action of SP-A on [IFN-γ+LPS]-activated macrophages is based on its capability to attenuate both inflammatory agents. Whereas the mechanism by which SP-A limits LPS-induced activation of macrophages was previously described, our study unravels a previously unknown mechanism by which SP-A/IFN-γ interaction controls inflammation in the alveolus. We demonstrate that SP-A binds to IFN-γ with high affinity, inhibiting IFN-γ recognition by its receptor on the cell surface of human alveolar macrophages. This mechanism allows SP-A to limit inflammation and maintain a tolerant lung environment in the steady state.

- SP-A enhances IL-4-dependent alternative activation and proliferation of alveolar macrophages, and this contributes to resolution of lung injury following *N brasiliensis* infection. SP-A acts via myosin18A receptor (Myo18A, *aka* SP-R210), whose expression in the macrophage cell surface was increased following IL-4 activation. In addition, we found that beyond the alveolus other secreted soluble defense collagens, such as C1q, also act through Myo18A receptor to promote type-2 immunity. C1q enhances IL-4-mediated macrophage alternative activation and proliferation in the peritoneal cavity promoting fibrosis in a model of peritoneal dialysis. We conclude that SP-A in the lung and C1q in the peritoneal cavity are tissue-specific factors needed to amplify the alternative activation and proliferation of IL-4-activated macrophages, with profound consequences for immunity to infection, tissue repair, and fibrosis.

- SP-A, through binding to Myo18A receptor, activates PI3K, and blockade of PI3K activity abrogated SP-A´s effects on IL-4 signalling. SP-A-dependent activation of PI3K and subsequent phosphorylation of its downstream effectors Akt and PKCζ amplifies IL-4-mediated macrophage proliferation and alternative activation. On

one side, SP-A sustained the PI3K-Akt signaling pathway triggered by IL-4, which is involved in macrophage proliferation. On the other hand, the SP-A/Myo18A/PI3K/PKCζ axis was involved in enhancing IL-4-dependent STAT6 activation, required for an alternative macrophage phenotype.

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